



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: <b>PCT/GB00/01558</b></p> <p>(22) International Filing Date: 20 April 2000 (20.04.00)</p> <p>(30) Priority Data:</p> <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">9909072.2</td> <td style="width: 30%;">20 April 1999 (20.04.99)</td> <td style="width: 40%;">GB</td> </tr> <tr> <td>09/311,817</td> <td>13 May 1999 (13.05.99)</td> <td>US</td> </tr> <tr> <td>2,271,451</td> <td>25 May 1999 (25.05.99)</td> <td>CA</td> </tr> </table> <p>(71) Applicant (for all designated States except US): <b>MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB).</b></p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): <b>HOLDER, Anthony [GB/GB]; MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA (GB). BIRDSALL, Berry [US/GB]; MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA (GB). FEENEY, James [GB/GB]; MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA (GB). MORGAN, William [US/GB]; MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA (GB). SYED, Shabih [GB/GB]; MRC National Institute for Medical Research,</b></p>	9909072.2	20 April 1999 (20.04.99)	GB	09/311,817	13 May 1999 (13.05.99)	US	2,271,451	25 May 1999 (25.05.99)	CA	<p>The Ridgeway, Mill Hill, London NW7 1AA (GB). UTHAIPBULL, Chairat [TH/TH]; Molecular Biology and Protein Engineering Laboratory, NCGEB (TH).</p> <p>(74) Agents: <b>MASCHIO, Antonio et al.; D. Young &amp; Co., 21 New Fetter Lane, London EC4A 1DA (GB).</b></p> <p>(81) Designated States: <b>AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</b></p> <p><b>Published</b>  <i>Without international search report and to be republished upon receipt of that report.</i></p>				
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<p>(57) Abstract</p> <p>A non-naturally occurring variant of a C-terminal fragment of a <i>Plasmodium</i> merozoite surface protein-1 (MSP-1) wherein said variant has (i) a reduced affinity, compared with a naturally occurring <i>Plasmodium</i> MSP-1<sub>19</sub>, for at least one first antibody capable of blocking the binding of a second antibody, which second antibody inhibits the proteolytic cleavage of <i>Plasmodium</i> MSP-1<sub>42</sub> and (ii) substantially the same affinity for at least one third antibody compared with said naturally occurring <i>Plasmodium</i> MSP-1<sub>19</sub>, which third antibody inhibits the proteolytic cleavage of <i>Plasmodium</i> MSP-1<sub>42</sub> is provided for use in an anti-malarial vaccine.</p>														

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VACCINEField of the Invention

5 The present invention relates to modified *Plasmodium* MSP-1 protein variants and their use in producing a vaccine against malaria. It also relates to a method for the rational design of suitable variants.

Background to the Invention

10

Malaria is a devastating disease that causes widespread morbidity and mortality in areas where it is transmitted by anopheline mosquitoes. In areas of high transmission young children and non-immune visitors are most at risk from this disease, which is caused by protozoa of the genus *Plasmodium*. In areas of lower or unstable transmission, epidemics  
15 of the disease can result and afflict individuals of all ages. The most dangerous form of malaria, responsible for much of the morbidity and most of the mortality, is caused by the species *Plasmodium falciparum*. It has been estimated that 2 billion people are at risk from malaria, with 200-300 million clinical cases and 1-2 million deaths each year.

20 The parasite has a complex life cycle in its human and mosquito hosts. In humans the stage of the life cycle which is responsible for the clinical symptoms of the disease occurs in the bloodstream. During this phase the parasite is largely hidden within host red blood cells. Here the parasite grows and multiplies. For example, within a red blood cell each *P. falciparum* parasite divides several times to produce approximately 20 new ones during  
25 a 48 hour cycle. At this point the red blood cell is burst open and the parasites (called merozoites at this stage) are released into the bloodstream. The merozoites must enter new red blood cells in order to survive and for the cycle of replication in the blood to continue. If the parasites do not manage to enter red blood cells they cannot survive for very long and are rapidly destroyed. Symptoms of malaria such as fever are associated with this  
30 cyclic merozoite release and re-invasion of red blood cells.

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There is an urgent need for a vaccine against malaria. There is no effective vaccine currently available. In addition, mosquito control by the spraying of residual insecticides is either becoming ineffective or considered to be unacceptable, and there is a very worrying spread of drug resistance within parasites. The rapid spread of drug resistance is worrying because compounds such as the cheap and once-effective chloroquine are no longer useful in many parts of the world, and there are few if any new drugs available that are both cheap and effective. Vaccines against microorganisms can be very cost effective and efficient ways to protect populations against infectious diseases.

Because of the complexity of the parasite's life cycle there are a number of points in its development within humans that could be the target of a protective immune response. It is known that with increasing age and exposure individuals do become immune to malaria, suggesting that protective responses do develop with time. Broadly speaking there are three types of vaccine strategy: to target the pre-erythrocytic stages, the asexual blood stage and the sexual stage. The pre-erythrocytic stages are the sporozoites that are injected by an infected mosquito when it takes a blood meal and the initial development of the parasite in the liver. The asexual blood stage is the infection and release of merozoites from red blood cells that occurs in a cyclic manner, and the stage responsible for the manifestation of the clinical symptoms. The sexual stage takes place in the mosquito's gut after it has ingested gametocytes in a blood meal and this initiates the infection of the insect to complete the cycle; a vaccine against the sexual stages would not protect the individual but could reduce transmission and therefore the incidence of malaria in a given human population.

During the asexual cycle in the blood the parasite is directly exposed to the host's immune system, and in particular to antibodies circulating within the bloodstream, only transiently: when merozoites are released by rupture of one cell and before they penetrate another. If there are specific antibodies that can bind to the surface of the parasite then it is possible that these antibodies will interfere with the ability of the parasite to invade a new red blood cell. In fact it has been shown that several monoclonal antibodies that recognise single epitopes on parasite surface proteins, are capable of neutralising the parasite and preventing the cycle of reproduction within red blood cells.



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One of the best characterised proteins on the surface of the merozoite is called merozoite surface protein 1 (MSP-1). MSP-1 is a large protein that varies in size and amino acid sequence in different parasite lines. It is synthesised as a precursor molecule of ~200 kDa by the intracellular parasite and located on the parasite's surface. During release of merozoites from red blood cells and the re-invasion of new erythrocytes the protein undergoes at least two proteolytic modifications. In the first modification as a result of a process called primary processing, the precursor is cleaved to four fragments of ~83, 30, 38 and 42 kDa that remain together as a complex on the merozoite surface. This complex also contains two other proteins of 22 kDa and 36 kDa derived from different genes. The complex is maintained by non-covalent interactions between the different subunits and is held on the merozoite surface by a glycosyl phosphatidyl inositol anchor, attached to the C-terminus of the 42 kDa fragment and inserted into the plasma membrane of the merozoite. At the time of merozoite invasion of an erythrocyte the C-terminal 42 kDa fragment is cleaved by a second proteolytic cleavage in a process called secondary processing. The result of secondary processing is that the entire complex is shed from the surface of the merozoite except for a C-terminal sub-fragment that consists of just under one hundred amino acids and which is carried into the newly invaded erythrocyte on the surface of the merozoite.

Based on sequence similarities, the structure of this small C-terminal fragment (called MSP-1<sub>19</sub>) was suggested to consist of two epidermal growth factor (EGF)-like domains (see sequence in Figure 1) (Blackman *et al.*, 1991). An EGF-like motif consists of a 45-50 amino acid sequence with a characteristic disulphide bonding pattern and such domains occur frequently in extracellular modular proteins of animals. In the MSP-1 C-terminal fragment each of the motifs contains six Cys residues proposed to form three disulphide bonds and each motif has a partial match to the EGF consensus (see Figure 1). However, because the degree of similarity is limited and since the pattern of its disulphide bonding is not known, the designation of the MSP-1 C-terminal fragment as comprised of EGF-like structures has been regarded as tentative. Other relatively divergent potential EGF-like sequences occur in Plasmodium proteins, but previous structure determinations have been confined to those from metazoan organisms (Campbell *et al.*, 1998).

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A number of studies have implicated MSP-1 as the target of a protective immune response. Although the goal of this work is to develop a malaria vaccine for use in humans, out of necessity most of this experimental work has been done either in model animal systems or *in vitro*. These include studies of the effect of specific antibodies on parasite invasion of erythrocytes *in vitro*, passive immunisation studies in rodent malaria models in laboratory mice and direct immunisation in both rodent and primate malaria models using either native protein (derived from the parasite) or recombinant protein expressed from parts of the MSP-1 gene in heterologous organisms. Sero-epidemiological studies have also showed a correlation between human antibody responses to parts of the MSP-1 molecule and protection against clinical disease. Much, but not all, of the work has focused on the immune response to the C-terminal MSP-1<sub>19</sub>. For example some monoclonal antibodies that recognise MSP-1<sub>19</sub> prevent red blood cell invasion in *in vitro* cultures (Blackman *et al.*, 1990). Interestingly, these antibodies that inhibit invasion also inhibit the secondary processing of the 42 kDa fragment, suggesting the mechanism by which they work is by steric hinderance of the protease responsible for secondary processing (Blackman *et al.*, 1994). Since secondary processing goes to completion during successful invasion. if it cannot occur then invasion is interrupted.

All of the work described above would suggest that MSP-1 and in particular polypeptides based on the C-terminal sequence that forms the 42 kDa or the MSP-1<sub>19</sub> region, should be very good candidates for malaria vaccine development. However, several studies have shown that the epitopes or binding sites for antibodies on MSP-1<sub>19</sub> require a correct polypeptide tertiary structure, and that this is destroyed by treatments that reduce the disulphide bonds that are postulated to be present between the cysteine residues present in MSP-1<sub>19</sub>. This limitation appears to have been overcome by the expression of recombinant protein in ways that allow antibodies that recognise the native parasite MSP-1 to bind. Other investigators have suggested that other parts of MSP-1 also have potential for inclusion in a vaccine, however the MSP-1 C-terminal fragment is currently the lead candidate for development of a vaccine against the blood stages of the malaria parasite (Diggs *et al.*, 1993; Stoute *et al.*, 1998).

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As stated above, every ~48 hours *P. falciparum* merozoites are released from the infected erythrocyte to re-invade new red blood cells and during this time they are exposed to the host's immune system. Therefore, the question arises as to how the parasite has evolved to avoid the potentially lethal effects of, for example, neutralising antibodies. In other infectious micro-organisms it is clear that there is a constant battle between the immune system and the micro-organism, and that sophisticated mechanisms have been evolved by micro-organisms to evade the immune response. For example antigenic variation and antigenic diversity are two mechanisms that involve presenting the immune system with "a moving target" such that even though an immune response to one variant of the micro-organism may kill that variant, new variants are produced that are at least partially or fully resistant to the immune response. In the case of malaria merozoites and in particular MSP-1 an alternative mechanism has been proposed whereby the binding of some antibodies ("blocking antibodies") can prevent the binding of neutralising antibodies and thereby allow the parasite to successfully invade a red blood cell even in the presence of neutralising antibodies (Guevara Patiño *et al.*, 1997). These blocking antibodies may be of two types, those against epitopes that are formed from amino acids that are distant in the linear primary sequence from the epitopes that are the target of neutralising antibodies, and those that are against epitopes that overlap with the epitopes of the neutralising antibodies. This represents a novel mechanism by which a parasite can evade an effective immune response, and unlike mechanisms based on antigenic polymorphism or diversity, it is not dependent upon amino acid sequence diversity.

Some monoclonal antibodies (mAbs) that bind to MSP-1<sub>19</sub> inhibit the proteolytic cleavage and erythrocyte invasion, suggesting that cleavage is a prerequisite for invasion (Blackman *et al.*, 1994). Other mAbs that bind to the MSP-1 C-terminal fragment do not inhibit processing or invasion but block the binding of the inhibitory neutralizing antibodies. Other antibodies that bind to MSP-1<sub>19</sub> neither inhibit nor block the binding of inhibitory antibodies. In the presence of blocking antibodies, inhibitory antibodies are ineffective and invasion proceeds. The balance between inhibitory and blocking antibodies induced by immunisation may be a critical factor in determining whether or not the immune response is effective in preventing invasion (Guevara Patiño *et al.*, 1997).

### Summary of the Invention

An object of the present invention is therefore to provide an effective vaccine against the malaria parasite based on variants of the *Plasmodium* MSP-1 protein. In designing such a vaccine, the following criteria should be met:

1. The amino acid sequence of the polypeptide to be used in the vaccine should contain epitopes that are the targets of, and can induce, neutralising antibodies.

2. The polypeptide should ideally not include amino acid sequences that only form epitopes for blocking antibodies.

3. If the polypeptide contains epitopes for both neutralising and blocking antibodies then it should be modified to remove the blocking antibody epitopes without affecting the neutralising epitopes.

To assist in the design of candidate vaccine polypeptides fulfilling these three criteria, it is important to determine the three-dimensional structure of the MSP-1 C-terminal fragment since this will help in mapping sites of antibody interactions with this fragment. We have therefore determined the solution structure of the MSP-1 C-terminal, including the pattern of disulphide bonding, using NMR techniques.

We have made amino acid substitutions in the sequence of MSP-1<sub>19</sub> that prevent the binding of individual blocking monoclonal antibodies, without affecting the binding of neutralising antibodies. By determining the 3-dimensional structure of MSP-1<sub>19</sub> we have identified where these antibody binding sites are located in the tertiary structure and this has allowed other amino acid substitutions to be made that have similar properties. We have shown that several substitutions, each affecting the binding of one or more blocking antibodies can be combined into a single molecule, and that these modified molecules continue to bind the neutralising antibodies but fail to bind any of the blocking antibodies. Such modified molecules are expected to be much more effective than the natural or wild-type protein structure at inducing a protective neutralising antibody response when used to

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immunise individuals as a malaria vaccine. In addition we have made other modifications in the primary structure of the molecule which do not affect the binding of the neutralising antibodies but which may contribute to increased immunogenicity of the molecule. The modified MSP-1<sub>19</sub> structures, either alone or coupled to other carriers, which may or may not contain other parts of MSP-1 to enhance the immunogenicity (for example a combination of the remainder of the MSP-1<sub>42</sub> kDa fragment with the modified MSP-1<sub>19</sub>) and provide additional T cell epitopes, would be more effective vaccines than equivalent structures that have not been modified in this way.

Accordingly, the present invention provides a non-naturally occurring variant of a C-terminal fragment of a *Plasmodium* merozoite surface protein-1 (MSP-1) wherein said variant has (i) a reduced affinity, compared with a naturally occurring *Plasmodium* MSP-1<sub>19</sub>, for at least one first antibody capable of blocking the binding of a second antibody, which second antibody inhibits the proteolytic cleavage of *Plasmodium* MSP-1<sub>42</sub> and (ii) substantially the same affinity for said second antibody compared with said naturally occurring *Plasmodium* MSP-1<sub>19</sub>.

Preferably, the *Plasmodium* MSP-1<sub>19</sub> and MSP-1<sub>42</sub> are *Plasmodium falciparum* MSP-1<sub>19</sub> and MSP-1<sub>42</sub>.

The first antibody is preferably selected from mAbs IE1, 2.2, 7.5, 9C8 and 111.4. The second antibody is preferably selected from mAbs 12.8, 12.10 and 5B1.

The present invention further provides a non-naturally occurring variant of a C-terminal fragment of a *Plasmodium* merozoite surface protein-1 (MSP-1) comprising an amino acid modification at any one of amino acid residues 14, 15, 27, 31, 34, 43, 48 and 53 of the *Plasmodium falciparum* MSP-1<sub>19</sub> amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

Preferably said modifications are substitutions selected from Gln14→Arg, Gln14→Gly, Asn15→Arg, Glu27→Tyr, Leu31→Arg, Tyr34→Ser, Tyr34→Ile, Glu43→Leu, Thr48→Lys and Asn53→Arg and their equivalents in other *Plasmodium* MSP-1<sub>19</sub>

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polypeptides. More preferably said substitutions are combinations of substitutions selected from [Glu27→Tyr, Leu31→Arg and Glu43→Leu], [Glu27→Tyr, Leu31→Arg, Tyr34→Ser and Glu43→Leu], [Asn15→Arg, Glu27→Tyr, Leu31→Arg and Glu43→Leu] and their equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

5

In a preferred embodiment, a variant MSP-1 polypeptide of the invention further comprises a mutation at Cys12 and/or Cys28 of the *Plasmodium falciparum* MSP-1<sub>19</sub> amino acid sequence shown as SEQ I.D. No. 1. Preferably such modifications are substitutions selected from Cys12→Ile and Cys28→Trp, and Cys12→Ala and  
10 Cys28→Phe.

Most preferably the substitutions are combinations selected from [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Glu43→Leu], [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Glu43→Leu, Asn53→Arg],  
15 [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Tyr34→Ser, Glu43→Leu, Asn53→Arg] and their equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

The present invention also provides a method for producing a *Plasmodium* MSP-1 variant  
20 for use in preparing a vaccine composition which method comprises modifying one or more amino acid residues of a *Plasmodium* MSP-1 C-terminal fragment such that the resulting derivative has (i) a reduced affinity, compared with a naturally occurring *Plasmodium* MSP-1<sub>19</sub>, for at least one first antibody capable of blocking the binding of a second antibody, which second antibody inhibits the proteolytic cleavage of *Plasmodium*  
25 MSP-1<sub>42</sub> and (ii) substantially the same affinity for said second antibody compared with said naturally occurring *Plasmodium* MSP-1<sub>19</sub>. In particular the method of the invention preferably comprises as a preliminary step, selecting a candidate amino acid residue by reference to a three dimensional NMR model structure, preferably as set out in Table 2. More specifically, the 3D model structure is used to select a surface exposed amino acid  
30 residue.) Advantageously, a further step is included of computer modelling the three dimensional structure of the variant to exclude polypeptides that do not fold correctly.

The present invention also provides a non-naturally occurring *Plasmodium* MSP-1 variant obtained by the method of the invention.

In a further aspect, the present invention provides a polynucleotide encoding a variant of the invention operably linked to a regulatory sequence capable of directing the expression of said nucleotide in a host cell. The polynucleotide may comprise a sequence which has been optimised for expression in the host cell. The host cell may be a *Pichia pastoris* cell. Also provided is a nucleic acid vector comprising a polynucleotide of the invention, including viral vectors, and a host cell comprising a nucleotide or vector of the invention.

In another aspect, the present invention provides a pharmaceutical composition comprising a variant of the invention, a polynucleotide of the invention or a vector of the invention together with a pharmaceutically acceptable carrier or diluent.

Preferably, the composition further comprises an immunogenic *Plasmodium* polypeptide or fragment or derivative thereof such as MSP-1<sub>33</sub> or a fragment or derivative thereof which may be covalently attached to the non-naturally occurring MSP-1<sub>19</sub>. It is preferred not to use wild-type MSP-1<sub>19</sub> sequences. The further immunogenic peptide may itself be derivatised in an analogous manner as described above for MSP-1<sub>19</sub>. Thus, epitopes present in the peptide may be identified and modified to prevent binding of blocking antibodies, without affecting the binding of neutralising antibodies. These epitopes may be capable of binding to antibodies which have similar properties to the first antibody described above, for example, in binding affinity. The further immunogenic peptide may comprise several such modifications in its amino acid sequence.

The present invention also provides a method for producing anti-MSP-1 antibodies which method comprises administering a polypeptide variant of the invention, or a polynucleotide of the invention or a vector of the invention to a mammal, typically a non-human mammal.

In a preferred embodiment, the present invention provides a method for producing polyclonal anti-MSP-1 antibodies which method comprises administering a polypeptide

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variant of the invention, or a polynucleotide of the invention or a vector of the invention to a mammal, typically a non-human mammal, and extracting the serum from said mammal. Also provided is an antibody produced by the said methods.

5 The polypeptides, nucleotides and vectors of the present invention may be used in methods of treating and/or preventing malaria caused by *Plasmodium* species, in particular *Plasmodium falciparum*. Accordingly, the present invention provides a method of inducing immunity against malaria induced by *Plasmodium falciparum* which comprises administering to a person in need of such immunity an effective amount of a variant, a  
10 polynucleotide or a vector of the invention.

Also provided is a method of immunizing a mammal, said method comprising administering an effective amount of a variant, a polynucleotide or a vector of the invention. In particular, said mammal is immunized against malaria. Preferably the  
15 mammal is a human.

The present invention also provides a method of treating a malaria infection in a human patient which comprises administering to the patient an effective amount of the pharmaceutical composition of the invention.

20

We further provide according to the present invention a nucleic acid encoding a *Plasmodium* MSP-1 polypeptide, in which the nucleic acid is optimised for expression in a heterologous host cell. Preferably, the heterologous host is a *Pischia pastoris* cell. The MSP-1 polypeptide may be selected from the group comprising an MSP-1<sub>42</sub> polypeptide  
25 comprising a sequence shown in Figures 2C and 2E, an MSP-1<sub>19</sub> polypeptide comprising a sequence shown in Figure 2C, and an MSP-1<sub>33</sub> polypeptide comprising a sequence shown in Figure 2E. The optimised nucleic acid may comprise a sequence selected from the sequences of Figure 2A, Figure 2B and Figure 2D. We further provide a vector comprising such a nucleic acid, a host cell comprising such a vector, and a pharmaceutical  
30 composition comprising such a nucleic acid or a vector, together with a pharmaceutically acceptable carrier or diluent. The pharmaceutical composition may further comprise an immunogenic *Plasmodium* polypeptide or fragment or derivative thereof.



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Detailed description of the invention

Although in general the techniques mentioned herein are well known in the art, reference  
5 may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual  
(1989) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley &  
Sons, Inc.

A. MSP-1 variant polypeptides

10 The variant MSP-1 polypeptides of the present invention will be described with reference  
to *Plasmodium falciparum* MSP-1 amino acid sequences. However, it should be  
appreciated that except where otherwise stated, all references to MSP-1 polypeptides  
include homologues of MSP-1 found in other *Plasmodium* species, such as *P. vivax*,  
15 *P. malariae* and *P. ovale* which all infect humans and *P. yoelii* which infects mice.

The variant MSP-1 polypeptides of the present invention are based on C-terminal  
fragments of the *Plasmodium falciparum* MSP-1<sub>42</sub> polypeptide shown as SEQ I.D. Nos. 2  
or 3. Such polypeptides will comprise some or all of the MSP-1<sub>19</sub> region (SEQ I.D. No.  
20 1), preferably at least substantially all of the domain 1 and/or domain 2 EGF-like  
sequences found in MSP-1<sub>19</sub> (approximately amino acids 1-47 and amino acids 48-96,  
respectively, of SEQ I.D. No. 1). It is particularly preferred to use regions that are  
conserved in most, more preferably all parasites of a single species to increase the  
effectiveness of the variant as a vaccine against a wide range of strains.

25 Variant MSP-1 polypeptides of the present invention comprise modifications to their  
primary amino acid sequence that reduce the ability of blocking antibodies to bind to the  
MSP-1 polypeptides. In addition, any modifications made should maintain epitopes  
recognised by neutralising antibodies such that the affinity of the neutralising antibodies  
30 for the MSP-1 variant is substantially the same as for naturally-occurring MSP-1  
polypeptides (such as an MSP-1<sub>42</sub> polypeptide having the sequence shown in SEQ I.D.  
Nos. 2 or 3). Some reduction in the binding of some neutralising antibodies may be

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tolerated since the primary objective is to inhibit the binding of blocking antibodies and it is likely that an effective reduction in the binding of blocking antibodies will compensate in terms of overall vaccine efficacy for a small reduction in neutralising antibody binding.

5 Neutralising antibodies in the context of the present invention are antibodies that inhibit malaria parasite replication. A variety of neutralising antibodies, polyclonal and monoclonal, are known in the art, including mAbs 12.8, 12.10 and 5B1 referred to in the Examples. The activity of neutralising antibodies can be determined in a variety of ways that have been described in the art. For example, a convenient assay method described in 10 Blackman *et al.*, 1994 involves using preparations of merozoites (Blackman *et al.*, 1993; Mrema *et al.*, 1982) to measure cleavage of MSP-1<sub>42</sub> into MSP-1<sub>33</sub> and MSP-1<sub>19</sub>. Briefly, freshly isolated merozoites are washed in ice-cold buffer and divided up into aliquots of about  $2 \times 10^9$  merozoites. A test antibody is added to each aliquot and the sample incubated at 37°C for 1 hour. The samples are then subjected to SDS-PAGE under non-reducing conditions on a 12.5% polyacrylamide gel, Western blotted and the blot probed 15 with antiserum to MSP-1<sub>33</sub>. In the control sample, two main bands are seen – one corresponding to MSP-1<sub>42</sub> and one lower molecular weight band corresponding to MSP-1<sub>33</sub>. Neutralising antibodies will reduce the amount of the lower molecular weight band as a result of inhibiting secondary proteolytic processing of MSP-1<sub>42</sub>.

20 This method is a particularly preferred method for assessing the efficacy of neutralising antibodies in the presence of antibodies believed to act as blocking antibodies. Where candidate competing blocking antibodies are to be tested, the merozoite sample is preincubated with a blocking antibody for 15 mins on ice prior to incubation with a 25 neutralising antibody at 37°C for 1 hour as described above. Thus blocking antibodies can readily be identified and/or characterised using such an assay method.

Other assay methods include merozoite invasion inhibition tests as described in Blackman *et al.*, 1990.

30 As discussed above, blocking antibodies are defined in the context of the present invention as antibodies that inhibit the binding of neutralising antibodies to MSP-1 but

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which do not themselves inhibit invasion of red blood cells by malaria parasites. Thus they "block" the neutralising function of the neutralising antibodies. A variety of blocking antibodies have been characterised in the art, including mAbs IE1, 2.2, 7.5 and 111.4 referred to in the Examples. As discussed above, blocking antibodies can conveniently be identified and/or characterised using assays that test their effect on neutralising antibody function.

Modifications that may be made to produce MSP-1 variants of the invention include substitutions, deletions and insertions. It is particularly preferred to use substitutions to minimise disruption of the secondary/tertiary structure of the polypeptide. Furthermore, particularly preferred substitutions are those that replace one class of amino acid with another class, such as an aliphatic non-polar residue with a charged polar residue. For example, the twenty naturally occurring amino acids may be divided into four main groups (aliphatic non-polar [G, A, P, I, L and V], polar un-charged [C, S, T, M, N and Q], polar charged [D, E, K and R] and aromatic [H, F, W and Y]) and it is preferred to replace an amino acid from one group with an amino acid from another group.

Other possibilities include replacing a positively charged side chain with a negatively charged side chain, replacing an amino acid with a large side chain with an amino acid with a smaller or no side chain (glycine), replacing a polar amino acid with a charged polar amino acid, replacing a large aromatic amino acid with an amino acid with a small side chain, and replacing cysteine residues that are involved in disulphide bonds.

Particularly preferred modifications are an amino acid modification at any one of amino acid residues 14, 15, 27, 31, 34, 43, 48 and 53 of the *Plasmodium falciparum* MSP-1<sub>19</sub> amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other *Plasmodium* MSP-1<sub>19</sub> polypeptides. These residues are all almost within the EGF-like domain 1. It is known that the epitopes of some antibodies contain amino acid sequences that are within EGF-like domain 2, therefore equivalent modifications may also be made in EGF-like domain 2. Preferred examples of modifications include the following substitutions Gln14→Arg, Gln14→Gly, Asn15→Arg, Glu27→Tyr, Leu31→Arg, Tyr34→Ser, Tyr34→Ile, Glu43→Leu, Thr48→Lys and/or Asn53→Arg and their equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

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It is especially preferred to carry out more than one modification, i.e. to use combinations of modifications, such as two or more or three or more. In a preferred embodiment, an MSP-1 variant of the invention comprises a combination of amino acid substitutions selected from [Glu27→Tyr, Leu31→Arg and Glu43→Leu], [Glu27→Tyr, Leu31→Arg, Tyr34→Ser and Glu43→Leu], [Asn15→Arg, Glu27→Tyr, Leu31→Arg and Glu43→Leu] and their equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

A particularly preferred combination further comprises a modification to Cys12 and/or Cys28 (and/or their equivalent residues in EGF-like domain 2) to disrupt the disulphide bond. Preferably such modifications are substitutions selected from Cys12→Ile and Cys28→Trp, and Cys12→Ala and Cys28→Phe.

Most preferably the substitutions are combinations selected from [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Glu43→Leu], [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Glu43→Leu, Asn53→Arg], [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Tyr34→Ser, Glu43→Leu, Asn53→Arg] and their equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

Substitutions are not confined to using naturally occurring amino acids – non-naturally occurring amino acid analogues may also be used, in particular where solid phase synthesis is to be used to chemically synthesise the variant, as opposed to recombinant technology.

Modifications to MSP-1 amino acid sequences may be carried out using standard techniques such as site-directed mutagenesis using the polymerase chain reaction. Alternatively, variants may be obtained by solid phase synthetic techniques.

To determine whether a variant MSP-1 polypeptide produced by modification of its primary amino acid sequence complies with the criteria specified above, the affinity of at least one neutralising antibody and at least one blocking antibody for the variant

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polypeptide compared with the naturally occurring MSP-1 sequence may be tested. Ideally more than one of each type of antibody should be used. for example two or three.

The ability of antibodies to bind to the variant and wild-type polypeptides may be determined using any one of a variety of methods available in the art for determining antibody-epitope binding. One such method, described in the Examples, involves the use of MSP-1 sequences expressed as fusion proteins with a protein tag such as glutathione-S-transferase (GST). These GST-fusion proteins are typically immobilised to a solid phase such as glutathione sepharose beads or a BIAcore sensor chip. Binding of antibodies, such as monoclonal antibodies, to the fusion proteins may be determined using standard techniques such as Western blotting and/or by labelling the antibodies with a radioactive label such as <sup>125</sup>I. The use of BIAcore technology allows easy quantitation of the results.

Preferably, the reduction in binding of at least one of the blocking antibodies tested is at least 50% compared to wild-type MSP-1, more preferably at least 75, 80 or 90%, typically as assessed using recombinantly expressed MSP-1 immobilised to a BIAcore sensor chip. By contrast, the binding of at least one, for example at least two or three, of the neutralising antibodies tested, more preferably at least half of the neutralising antibodies tested, more preferably substantially all of the neutralising antibodies tested is reduced by less than 50%, more preferably less than 25%. The number of neutralising antibodies that need be tested to confirm compliance with the test criteria will not typically exceed from three to five different antibodies (three antibodies are used in the Examples). In a particularly preferred embodiment the binding of at least one neutralising antibody is increased by at least 10%.

The results given in Table 2 in the Examples provide partial guidance to the skilled person as to which residues may be modified to produce a variant MSP-1 of the invention. However, the provision herein for the first time of the three dimensional solution structure of MSP-1<sub>19</sub> provides the skilled person with further detailed guidance as to which residues may be altered. In particular, epitopes are expected to be exposed to the aqueous environment on the exterior of the MSP-1<sub>19</sub> fragment. Consequently, the precise structural information provided which teaches the position of surface exposed amino acids

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allows the skilled person to target those residues for modification. This data is given in Tables A/B and has also been submitted to the Protein Data Bank (PDB Accession no. 1CEJ). It enables the skilled person to identify the precise location of individual amino acids in the three dimensional structure. Typically, the data is loaded into suitable software, well-known in the art such as Insight II, MOLSCRIPT GRAS P and RASMOL.

Further, knowing the location of a modification in the 3-dimensional structure which affects the binding of a blocking antibody without affecting the binding of the neutralising antibodies, it is possible to identify other residues that are on the surface and in the vicinity of the original modification and which may be easily modified to further improve the properties of a modified protein. These residues may be in either the first or the second EGF-like motifs or in the sequence between them. Since it is known that an antibody binding site can encompass a volume that corresponds approximately to the range of 5 to 8 amino acids, it is clear that modifications of these adjacent residues may also affect the affinity of the protein for the blocking antibodies. Once an adjacent amino acid has been identified it can be modified according to the principles outlined above and the contribution of the modification to the overall antigenicity and immunogenicity of the protein, either alone or in combination with other modifications, can be assessed. Those changes that contribute to a reduced affinity for the blocking antibodies, without a substantial affect on binding of the neutralising antibodies can be incorporated into the improved protein. This can be a reiterative process.

In addition, the 3D NMR structure will enable the skilled person to carry out preliminary computer modelling studies of MSP-1<sub>19</sub> variants with specific modifications so that, for example variants that cannot fold properly may be discarded. This will assist in minimising the number of candidate MSP-1<sub>19</sub> variants that need be tested.

Thus the present invention also provides a computer readable medium having stored thereon a model of the MSP-1<sub>19</sub> NMR structure. In a preferred embodiment, said model is built from all or some of the NMR data shown in Tables A and B.

Variants of the present invention may optionally include additional MSP-1 sequences, in

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particular regions of the MSP-1<sub>33</sub> region of MSP-1<sub>42</sub> to confer additional immunogenicity to the variant. Furthermore, additional sequences known to contain and promote T cell responses are advantageously included (i.e. T cell epitopes). Other modifications may also be made that increase immunogenicity such as modifications that alter the pathway of antigen processing and presentation.

Polypeptide variants of the invention are typically made by recombinant means, for example as described below. However they may also be made by synthetic means using techniques well known to skilled persons such as solid phase synthesis. Proteins of the invention may also be produced as fusion proteins, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and  $\beta$ -galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the immunogenicity of the MSP-1 variant.

Polypeptides of the invention may be in a substantially isolated form. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as substantially isolated. A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the polypeptide in the preparation is a polypeptide of the invention.

## B. Polynucleotides and vectors

As discussed above, the variants of the present invention may be produced recombinantly using standard techniques. Thus, the present invention also provides a polynucleotide encoding a polypeptide MSP-1 variant of the invention. Polynucleotides of the invention may comprise DNA or RNA. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to

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oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art.

5 Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of the invention. It will be understood by a skilled person that numerous different polynucleotides can encode the same polypeptide as a result of the degeneracy of the genetic code.

10 Polynucleotides of the invention comprise can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host  
15 cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect Sf9 cells. The host cell may be a methylotrophic yeast such as *Pichia pastoris*.

20 The coding sequence of natural or variant MSP polypeptides (including the polypeptide of the invention) may be modified for optimal expression in a host cell. For example, secondary modification such as N-glycosylation may be prevented by removal of sequences necessary for such modification. The sequence of the polypeptide may alternatively or in addition be modified with respect to codon usage for optimal expression  
25 in the host cell. Methods of mutagenising a sequence are known in the art; alternatively, the modified coding sequence may be generated by means of PCR gene assembly using overlapping synthetic oligonucleotides (Stemmer et al., 1995; Withers-Martinez et al., 1999).

30 Preferably, a polynucleotide of the invention in a vector is operably linked to a regulatory sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a



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juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

5

Such vectors may be transformed or transfected into a suitable host cell using standard techniques above to provide for expression of a polypeptide of the invention. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions to provide for expression by the vector of a coding  
10 sequence encoding the polypeptides, and optionally recovering the expressed polypeptides.

The vectors may be for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and  
15 optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell. The vector may also be adapted to be used *in vivo*, for example in a method of gene therapy.

20

Promoters/enhancers and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. For example, prokaryotic promoters may be used, in particular those suitable for use in *E. coli* strains (such as *E. coli* HB101 or DH5 $\alpha$ ).

25

When expression of the polypeptides of the invention is carried out in mammalian cells, either *in vitro* or *in vivo*, mammalian promoters may be used. Tissue-specific promoters may also be used. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the promoter of rous sarcoma virus  
30 (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, herpes simplex virus promoters or adenovirus promoters. All these promoters are readily available in the art.

### C. Administration

The variant MSP-1 polypeptides of the present invention and nucleic acid molecules may  
5 be used to treat or prevent malaria in animals, specifically humans.

The polypeptides of the invention may be administered by direct injection. Preferably the  
polypeptides are combined with a pharmaceutically acceptable carrier or diluent to  
produce a pharmaceutical composition. Suitable carriers and diluents include isotonic  
10 saline solutions, for example phosphate-buffered saline. The composition may be  
formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or  
transdermal administration. Typically, each polypeptide is administered at a dose of from  
0.01 to 30 µg/kg body weight, preferably from 0.1 to 10 µg/kg, more preferably from 0.1  
to 1 µg/kg body weight. It is also possible to use antibodies prepared using the  
15 polypeptides of the invention, as described below, in treating or preventing *Plasmodium*  
infection. Neutralising antibodies, or fragments thereof which retain specificity for  
*Plasmodium* antigens, can be administered in a similar manner to the polypeptides of the  
invention.

20 The polynucleotides of the invention may be administered directly as a naked nucleic acid  
construct. When the expression cassette is administered as a naked nucleic acid, the  
amount of nucleic acid administered is typically in the range of from 1 µg to 10 mg,  
preferably from 100 µg to 1 mg.

25 Uptake of naked nucleic acid constructs by mammalian cells is enhanced by several  
known transfection techniques for example those including the use of transfection agents.  
Example of these agents include cationic agents (for example calcium phosphate and  
DEAE-dextran) and lipofectants (for example lipofectam<sup>TM</sup> and transfectam<sup>TM</sup>).  
Typically, nucleic acid constructs are mixed with the transfection agent to produce a  
30 composition.

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Alternatively, the polynucleotide may be administered as part of a nucleic acid vector, including a plasmid vector or viral vector, such as a vaccinia virus vector. When the polynucleotide of the invention is delivered to cells by a viral vector of the invention, the amount of virus administered is in the range of from  $10^3$  to  $10^{10}$  pfu, preferably from  $10^5$  to  $10^8$  pfu, more preferably from  $10^6$  to  $10^7$  pfu. When injected, typically 1-10  $\mu$ l of virus in a pharmaceutically acceptable suitable carrier or diluent is administered.

Preferably the delivery vehicle (i.e. naked nucleic acid construct or viral vector comprising the polynucleotide for example) is combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

#### D. Preparation of Vaccines

Vaccines may be prepared from one or more polypeptides of the invention. They may also include one or more immunogenic *Plasmodium* polypeptides known in the art. Thus a vaccine of the invention may comprise one or more polypeptides of the invention and optionally, one or more polypeptides selected from, for example, the asexual blood stage proteins: apical merozoite antigen-1, erythrocyte binding antigen 175, erythrocyte membrane protein-1; the hepatic stage proteins: liver stage antigens 1 and 3; the sporozoite stage proteins: circumsporozoite protein, thrombospondin related adhesive protein; and the sexual stage proteins Pfs25 and Pfs28 polypeptides and immunogenic fragments thereof. Preferably, the other immunogenic *Plasmodium* polypeptides known in the art do not contain wild type MSP-1<sub>19</sub> sequences.

The preparation of vaccines which contain an immunogenic polypeptide(s) as active

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ingredient(s), is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion. The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic polypeptide containing an MSP-1 antigenic sequence resulting from administration of this polypeptide in vaccines which are also comprised of the various adjuvants.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1% to 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%. Where the vaccine composition is

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lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is preferably effected in buffer.

Capsules, tablets and pills for oral administration to a patient may be provided with an enteric coating comprising, for example, Eudragit "S", Eudragit "L", cellulose acetate, cellulose acetate phthalate or hydroxypropylmethyl cellulose.

The polypeptides of the invention may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric and maleic. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine and procaine.

#### E. Dosage and Administration of Vaccines

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The quantity to be administered, which is generally in the range of 5  $\mu$ g to 250  $\mu$ g of antigen per dose, depends on the subject to be treated, capacity of the subject's immune system to synthesise antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered may depend on the judgement of the practitioner and may be peculiar to each subject.

The vaccine may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1 to 4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the need of the individual and be

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dependent upon the judgement of the practitioner.

In addition, the vaccine containing the immunogenic MSP-1 antigen(s) may be administered in conjunction with other immunoregulatory agents, for example, immunoglobulins.

F. Preparation of antibodies against the polypeptides of the invention

The variant MSP-1 polypeptides prepared as described above can be used to produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide bearing an MSP-1 epitope(s). Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to an MSP-1 epitope contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art.

Monoclonal antibodies directed against MSP-1 epitopes in the polypeptides of the invention can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against MSP-1 epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

The polypeptides of the invention can also be used to select for human monoclonal antibodies using the variable regions of immunoglobulin heavy and light chains cloned in the form of a phage display library, preferably from individuals who have been previously exposed to a natural malaria infection.

Antibodies, both monoclonal and polyclonal, which are directed against MSP-1 epitopes are particularly useful in diagnosis, and those which are neutralising are useful in passive

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immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotypic antibodies. Anti-idiotypic antibodies are immunoglobulins which carry an "internal image" of the antigen of the infectious agent against which protection is desired.

5 Techniques for raising anti-idiotypic antibodies are known in the art. These anti-idiotypic antibodies may also be useful for treatment of *Plasmodium* infections, as well as for an elucidation of the immunogenic regions of MSP-1 antigens. It is also possible to use fragments of the antibodies described above, for example, F(ab')<sub>2</sub>, Fab, Facb and scFv fragments.

10 It should be appreciated that features from various sections, aspects and embodiments of the invention as described above are generally equally applicable to other sections, aspects and embodiments *mutatis mutandis*.

15 The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention. The Examples refer to the Figures. In the Figures:

20 Detailed Description of the Figures

Figure 1 - MSP-1 sequences aligned according to the EGF-like motif consensus. Top sequence: *P. falciparum* (SWISS-PROT MSP1 PLAFW). Second sequence: *P. vivax* Belem strain (PIR A45604). Third sequence: human EGF (PDB 1egf). Fourth sequence: 25 EGF-like domain consensus (Prosite EGF1). Bottom sequence: 14 residue EGF core region used for structure alignment in Figure 6. Black highlighting indicates conserved residues of the EGF-like domain. Dark shading shows hydrophobic residues at the EGF-module pair interface in the *P. falciparum*, and corresponding conserved residues in the *P. vivax* sequence.

30 Figure 2 - Sample of multidimensional heteronuclear NOESY experiments showing planes containing NOE connections to the MSP-1 C-terminal fragment Lys35 NH proton. Top: <sup>13</sup>C (D4) and <sup>1</sup>H(D3) plane from the 4D-[<sup>13</sup>C]-HMQC-NOESY-[<sup>15</sup>N]-HSQC

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experiment, taken at the chemical shift values of Lys35 NH in  $^{15}\text{N}$ (D2) and  $^1\text{H}$ (D1). Bottom: strip from the 3D [ $^{15}\text{N}$ ]-NOESY-HSQC at the  $^1\text{H}$  chemical shift value of Lys35 NH (vertical axis, D1) taken at the plane of its  $^{15}\text{N}$  (D3) value. The horizontal  $^1\text{H}$  axis is aligned with that of the top spectrum. The weak cross-peaks at 2.72 and 3.01 ppm in the 3D spectrum do not show corresponding cross-peaks in the 4D spectrum because of the lower signal-to-noise ratio in the latter. These peaks have been assigned as the cross-peaks between Lys35 NH and Asn44  $\text{H}_{\beta 2}$  (2.72 ppm), and Cys30  $\text{H}_{\beta 3}$  and/or Cys41  $\text{H}_{\beta 2}$  (3.01 ppm).

10 Figure 3 - Stereo drawing showing the backbone C, N,  $\text{C}_\alpha$  atoms of the 32 refined structures in the final ensemble. The domain-1 is on the left (red), with domain-2 on the right (green), and both the N- and C-termini are near the bottom.

Figure 4 - MOLSCRIPT picture of the most representative model of the ensemble, 15 showing the backbone  $\text{C}_\alpha$  trace, antiparallel  $\beta$ -sheet elements, and disulphide bridges ( $\text{S}_\gamma$  atoms in yellow). Domain-1, red; Domain-2, green.

Figure 5 - Alignment of typical EGF-like family members with the fitpdb program, using the 14 amino acid "reduced core" consensus (Bersch *et al.*, 1998) (see Figure 1). The aligned backbone segment in each structure is white. The structures are aligned relative to 20 the most representative structure of the group (factor Xa), with increasing divergence from left to right. Numbers indicate the rmsd value of the aligned C, N,  $\text{C}_\alpha$  atoms. PDB identification codes: factor Xa (crystal structure), 1hcg; Complement C1r component, 1apq (14<sup>th</sup> model); human EGF, 1egf (11<sup>th</sup> model); fibrillin-1, domains-32 and -33\_1emn (minimized average structure); transforming growth factor- $\alpha$ , 2tgf (minimized average structure); MSP-1 domains-1 and -2, this study.

Figure 6 - Backbone ribbon view of fibrillin-1 versus MSP-1 EGF module pair arrangements. Fibrillin-1 (1emn) cyan (domain-32) and magenta (domain-33) (Downing 30 *et al.*, 1996); MSP-1 domain-1 (yellow) and domain-2 (green). Structures were aligned as in Figure 6 by the core consensus of the N-terminal domain of each pair. The bound  $\text{Ca}^{2+}$  ions in the fibrillin-1 structure are shown as magenta spheres.



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Figure 7 - Two views, a and b, (rotated 180° about the y-axis) of the electrostatic potential surface of the MSP-1 EGF module pair, calculated with GRASP. Red indicates negative charge, blue indicates positive charge, and white is neutral. The orientation of the views is shown by the adjacent worm diagrams.

Figure 8 - CPK model of the MSP-1 C-terminal fragment, showing the location of some mutations that affect binding of monoclonal antibodies. Domain-1 is towards the top and right sides, and domain-2 towards the bottom left.

Figure 9 - Examples of the binding of monoclonal antibodies to GST-MSP-1<sub>19</sub> detected by Western blotting. The binding of each monoclonal antibody to protein based on the wild type sequence and to proteins containing modified sequences is shown. The monoclonal antibodies are shown across the top. On the left is shown the proteins: WT, wild type sequence; 22, Leu22 to Arg; 26, Glu26 to Ile; 15, Asn15 to Arg; 27, Glu27 to Tyr; 31, Leu31 to Arg; 43, Glu43 to Leu; 27+31+43, Glu27 to Tyr and Leu31 to Arg and Glu43 to Leu; 15+27+31+43, Asn15 to Arg and Glu27 to Tyr and Leu31 to Arg and Glu43 to Leu.

Figure 10 - The binding of monoclonal antibodies to GST-MSP-1<sub>19</sub> detected by BIAcore analysis. The binding of each monoclonal antibody is normalised to 100% binding to protein based on the wild type sequence and the binding of proteins containing modified sequences is expressed as a percentage of this. WT, wild type sequence; 15, Asn 15→Arg; 26, Glu26→Ile; 27, Glu27→Tyr; 31, Leu31→Arg; 34, Tyr34→Ser; 43 Glu43→Leu.

Figure 11 - The binding of monoclonal antibodies to GST-MSP-1<sub>19</sub> containing multiple modifications detected by BIAcore analysis. The binding of each monoclonal antibody is normalised to 100% binding to protein based on the wild type sequence and the binding of proteins containing modified sequences is expressed as a percentage of this. WT, wild type sequence; The combinations contain 3 mutations [27+31+43], or 4 mutations ([27+31+34+43] and [15+27+31+43]), at each site the changes are those identified in Figure 10.

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Figure 12 – Identification of blocking antibodies using a competitive binding assay and immobilised wild type GST-MSP-1<sub>19</sub>. The ability of antibodies to compete with the binding of mAbs 12.8 and 12.10 to GST-MSP-1<sub>19</sub> was measured using BIAcore analysis. Individual antibodies (x-axis) were bound to the antigen and then the amount of either 12.8 or 12.10 (inhibitory mAb) that could subsequently bind was quantified. The amount of binding is presented as a percentage of the total amount of either 12.8 or 12.10 bound in the absence of pre-incubation with another antibody.

Figure 13 - Antibodies induced by immunisation with a modified recombinant MSP-1<sub>19</sub> assayed for their ability to inhibit secondary processing. Washed 3D7 merozoites were either analysed directly without incubation (0 h) or incubated for 1 hour at 37°C in the presence of no serum (no serum), 1 mM PMSF as a control for complete inhibition, normal rabbit sera (normal serum), or serum from a rabbit immunised with the 15+27+31+43 modified protein (immune serum), all at 1:10 dilution in reaction buffer. The level of MSP-1<sub>33</sub> released into the supernatant as a results of secondary processing was measured using an ELISA method and is represented by Absorbance at 492nm.

Figure 14. *Pichia pastoris* codon preference table used for input to the CODOP program.

Figure 15. DNA and protein sequences for the optimized synthetic MSP-142 gene. A: Complete sequence designed for optimum codon usage and expression in *P. pastoris*. B: Sequence of the synthetic MSP-119 construct in the expression vector pPIC9K-HXa. Uppercase letters: vector sequences, including the His<sub>6</sub> tag and factor Xa cleavage site (IEGR). Lowercase letters: synthetic MSP-119 coding sequence. The cloned sequence in located at the SnaBI restriction site of the pPIC9K sequence. C: Expressed protein sequence of the synthetic MSP-119 construct. The sequence shown is produced as a fusion to the pPIC9K  $\alpha$ -factor secretion signal, following the kex2/STE13 processing sites. The synthetic MSP-119 is in bold-face type. D: Sequence of the MSP-133 construct. The cloned sequence is located at the SmaI site of the pUC118 vector. E: Predicted protein sequence of the synthetic MSP-133 construct translation product.

Figure 16. Gene assembly PCR reactions for the MSP-133 and MSP-119 sequences. Reaction 1: 10  $\mu$ L aliquots of the assembly reactions. Reaction 2: 20  $\mu$ L aliquots of the

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amplification reactions. The N-terminal and middle fragments were subsequently spliced together to form the MSP-133 synthetic construct. The C-terminal fragment synthesis reactions produced the optimized MSP-119 construct.

5 Figure 17. Expression of the synthetic MSP-119 protein in *P. pastoris*. Lanes 1-6: trichloroacetic acid precipitates of secreted recombinant protein from culture supernatants, without further purification (5  $\mu$ L each). Samples from duplicate cultures of three independent transformants. Lane 8,9: purified, deglycosylated MSP-119 produced from the original *P. falciparum* sequence. Lane 7,10: NOVEX molecular weight markers.

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Figure 18. A:  $\{^1\text{H}/^{15}\text{N}\}$ -HSQC spectrum of the protein (2.5 mM) expressed from the optimized synthetic MSP-119 gene. B: Control  $\{^1\text{H}/^{15}\text{N}\}$ -HSQC of deglycosylated protein (2.2 mM) expressed from the original *P. falciparum* sequence (Morgan et al., 1999).

## 15 Examples

### **Materials and Methods**

#### Protein expression and stable-isotope labelling for NMR

20

The coding sequence of the MSP-1 C-terminal fragment was cloned by polymerase chain reaction with Vent polymerase (New England Biolabs) from a plasmid containing the *Plasmodium falciparum* strain T9/94 fragment (Blackman *et al.*, 1991), using primers that included codons for a 6 residue N-terminal His tag (CACCATCATCATCAC), and  
25 inserted into the *Sna*BI restriction site of the pPIC9K vector (Invitrogen). The sequence corresponds to residues 1526-1621 of the SWISS-PROT entry MSP1 PLAFW (accession number P04933). This produced an  $\alpha$ -factor fusion protein with the sequence ...KR/EA/EA/YHHHHHHNISQ....SSSN. where the slashes indicate *kex2* and *STE13* processing sites. High copy number transformants of the methylotrophic yeast  
30 *Komagataella (Pichia) pastoris* protease-deficient strain SMD1168 (*his4 pep4*) were isolated by screening for high G418 resistance (Clare *et al.*, 1995).

A Mut<sup>-</sup> transformant was grown at 29.4 °C in a shaker-incubator in buffered minimal

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medium (100 mM potassium phosphate, pH 6.0, yeast nitrogen base (0.34 % w/vol) (DIFCO: YNB without amino acids and without  $(\text{NH}_4)_2\text{SO}_4$ ), biotin ( $4 \times 10^{-5}$  % w/vol), Sigma antifoam 289 (0.01 % vol/vol), and carbon and nitrogen sources as described below. Unlabelled samples were initially grown in medium containing 1 % w/vol  $(\text{NH}_4)_2\text{SO}_4$  and 1 % w/vol glycerol, and induced by transfer to medium containing 0.5 %  $\text{CH}_3\text{OH}$  as the carbon source. Labelled samples were initially grown in medium containing 0.2 % w/vol  $^{15}\text{N}$ - $(\text{NH}_4)_2\text{SO}_4$  (Isotech), and 0.5 % w/vol glucose or  $^{13}\text{C}_6$ -glucose (Isotech), and induced by transfer to medium containing as carbon source 0.5 % w/vol  $\text{CH}_3\text{OH}$  or  $^{13}\text{C}$ - $\text{CH}_3\text{OH}$  (Isotech). The initial cultures were grown in 150 ml to a density of  $\sim 10 \text{ OD}_{600}$ , then harvested and resuspended in methanol medium at 1  $\text{OD}_{600}$  in a volume of 1.5 L. Methanol-induced cultures were grown for 4 d, with daily addition of 7.5 ml  $\text{CH}_3\text{OH}$  or  $^{13}\text{C}$ - $\text{CH}_3\text{OH}$ , to a final density of  $\sim 18 \text{ OD}_{600}$ . This protocol produced a maximum yield of 24 mg/L of purified,  $^{13}\text{C}/^{15}\text{N}$  uniformly labelled protein at the final stage (see below). The YNB-based medium produced about 3-fold higher yields than the FM22 medium (Laroche *et al.*, 1994), for stable-isotope labeling of the MSP-1 C-terminal fragment.

Cells were removed by low-speed centrifugation, protease inhibitors added (COMPLETE<sup>TM</sup> tablets, Boehringer-Mannheim; 1 tablet/500 ml supernatant), and the supernatant was filter-sterilized. The supernatant was concentrated  $\sim 20$ -fold by ultrafiltration in a stirred cell (Amicon, YM3 membrane) at 4 °C. The pH was adjusted to 7.25 with KOH, and the partially N-glycosylated MSP-1 fragment was deglycosylated for 72 h at 37 °C with 5000 U PNGaseF (New England Biolabs). The carbohydrate was completely removed (as shown by electrophoresis and mass spectrometry), with the Asn 1 residue presumably converted to Asp in the process. The supernatant was clarified by low-speed centrifugation, 5 M NaCl was added to a final concentration of 0.3 M, and the sample was applied to a 2 ml Ni-NTA affinity column (QIAGEN), washed, and eluted with 250 mM imidazole according to manufacturer's instructions. The eluate was dialyzed against 50 mM sodium phosphate (pH 6.5), 50 mM NaCl, and then passed through a 1 ml Hi-Trap Q anion exchange resin (Pharmacia) to remove misfolded MSP-1 that bound to the column. The MSP-1 fragment was characterized by Western blotting and electrospray mass spectrometry (data not shown). Two principal species of mass 11607 and 11807 Da were observed corresponding to the expected fragment, as well as a

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fragment with an additional N-terminal Glu - Ala dipeptide resulting from incomplete STE13 processing of the  $\alpha$ -factor secretion signal.

Samples for NMR experiments were prepared in either 90 % H<sub>2</sub>O/10 % D<sub>2</sub>O with 0.01 % w/vol NaN<sub>3</sub> or 100 % D<sub>2</sub>O, 50 mM sodium phosphate, 100 mM NaCl at pH 6.5, (pH uncorrected for deuterium isotope effects), at a concentration of 2.1 to 2.6 mM in 0.6 ml. Protein concentration was measured by UV absorbance at 280 nm, using a calculated molar extinction coefficient of 5220 liter mol<sup>-1</sup> cm<sup>-1</sup>. The protein was demonstrated to be monomeric by equilibrium ultracentrifugation of a 0.12 mM sample in the above buffer at 293 K.

#### NMR experiments and data processing

Most of the experiments were performed at 298 K, using Varian Unity and Unity-Plus spectrometers operating at 600 MHz and 500 MHz respectively. Details of the multidimensional experiments (Clore & Gronenborn, 1998) and acquisition parameters used for resonance assignments and structure determination are given in Tables A/B and have been submitted to the Protein Data Bank database (PDB Accession No 1CEJ).

All spectra were processed using Felix 95.0 or 97.0 (Biosym/MSI) using a 90 degree- or 72 degree-shifted sinebell-squared window function. Dimensions, zero-filling, and linear prediction details are summarized in Tables A/B and in the submission to the BioMagResBank. Four dimensional and interleaved spectra were processed in Felix using macros written in-house.

*Signal assignments:* Sequential assignments were made based on connectivities established primarily by CBCA(CO)NH and CBCANH experiments on uniformly <sup>13</sup>C/<sup>15</sup>N labelled protein. Side chain-spin system assignments were made on the basis of data from <sup>13</sup>C/<sup>1</sup>H -HCCH-TOCSY experiment correlated with information from <sup>15</sup>N/<sup>1</sup>H-TOCSY-HSQC and <sup>15</sup>N/<sup>1</sup>H-NOESY-HSQC, and HNHA and HNHB experiments. Assignments were obtained for <sup>1</sup>H, <sup>15</sup>N and aliphatic-<sup>13</sup>C signals for 98% of side-chains and 96% of backbone amide groups. The list of assignments is given in Tables A/B and in the

submission to the Protein Data Bank database (PDB Accession No 1CEJ). The  $^{15}\text{N}\{^1\text{H}\}$  heteronuclear NOE experiment was carried out as described previously (Kay *et al.*, 1989; Polshakov *et al.*, 1997).

5 *Distance Restraints:* NOE- and ROE-derived distance restraints between backbone and side chain amide protons were obtained primarily from the 3D  $^{15}\text{N}$ -NOESY-HSQC,  $^{15}\text{N}$ -ROESY-HSQC, and 4D  $^{13}\text{C}$ -HMQC-NOESY- $^{15}\text{N}$ -HSQC experiments. Aliphatic to aliphatic proton distance restraints were obtained from a 4D  $^{13}\text{C}$ -HMQC-NOESY- $^{13}\text{C}$ -HSQC experiment. A 3D  $^{13}\text{C}$ -HMQC-NOESY experiment in  $\text{D}_2\text{O}$  was used to identify  
10 aliphatic to aromatic proton NOEs and 2D NOESY experiments were used to measure aromatic to aromatic proton NOEs. Crosspeaks were quantified by volume integration in Felix for 2D and 3D experiments and for the 4D  $^{13}\text{C}$ -HMQC-NOESY- $^{15}\text{N}$ -HSQC experiment, and from peak height measurements in the 4D  $^{13}\text{C}$ -HMQC-NOESY- $^{13}\text{C}$ -HMQC spectra. Crosspeaks were classified as strong, medium and weak and these were  
15 assigned to distance restraints of 0 - 2.8, 0 - 3.6, and 0 - 5.5 Å. Restraints from backbone amide signals were initially treated in this manner, and then recalibrated more precisely using 3D- $^{15}\text{N}$ -ROESY-HSQC data into four classes involving maximum distances of 2.6, 3.1, 3.6, and 4.1 Å. Restraints to groups of equivalent or non-stereoassigned protons were treated by  $r^{-6}$  summation. Most intraresidue distances ( $\text{HN-H}_\beta$  and  $\text{H}_\alpha\text{-H}_\beta$ ) were converted  
20 to  $\chi_1$  angle restraints as described below and these distance restraints were not included in the final list.

*Dihedral Angle Restraints:*  $\chi_1$  angles and stereospecific assignments of  $\beta$ -methylene protons were obtained using the grid-search program AngleSearch, with coupling constant  
25 and intraresidue ROE distance information (Polshakov *et al.*, 1995). The coupling constant information was provided by HNHB and HN(CO)HB spectral intensities for  $^3\text{J}(\text{HN-H}_\beta)$  and  $^3\text{J}(\text{CO-H}_\beta)$ , and intraresidue distances ( $\text{HN-H}_\beta$ ,  $\text{H}_\alpha\text{-H}_\beta$ ) were obtained from 3D  $^{15}\text{N}$ -ROESY-HSQC and 2D ROESY ( $\text{D}_2\text{O}$ ) experiments.  $^3\text{J}(\text{HN-H}_\alpha)$  coupling constants were obtained from the HNHA experiment. Residues with positive  $\phi$  angles (ca.  
30  $+60$  degrees) were identified by large intraresidue  $\text{H}_\alpha$  crosspeak intensities in the HN(CO)HB experiment, and  $\gamma$  angles near  $-60^\circ$  degrees from strong  $\text{H}_{\alpha(i-1)}$  crosspeaks in the HNHB experiment. Ile and Leu  $\chi_2$  angles and Leu  $\delta$  stereoassignments were derived

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from the LRCH experiment. Minimum ranges of 40 degrees ( $\chi_1, \chi_2$ ) and 50 degrees ( $\phi, \psi$ ) were used to account for errors and local dynamic effects on the coupling constants.

*Disulphide Bonding Pattern:* An initial set of 20 structures was calculated by simulated annealing using approximately 550 unambiguous NOE-derived distance restraints and 36  $\chi_1$  and  $\phi$  dihedral angle restraints but with no hydrogen bonding or disulphide bond constraints. The Cys - Cys  $S_\gamma$  distances in these structures were examined in order to establish the probable bonding pattern. Prior to the calculations, the formation of disulphide bridges for 4 Cys residues (Cys12 - Cys28, Cys78 - Cys92) was already established with high probability by the observation of  $H_\beta$ - $H_\beta$  NOEs between these pairs of Cys residues. Examination of the initial structures confirmed these disulphide bridges and also indicated a disulphide bridge between residues Cys30 and Cys41. The third disulphide bridge in domain-1 (Cys7 - Cys18) could thus be assigned by default, although the structure of the N-terminus was not well-defined by the NMR data. The best six structures in terms of total X-PLOR energy and violations indicated that the average Cys - Cys  $S_\gamma$  distance was lowest for the disulphide bonding pattern [1-3, 2-4, 5-6] in each domain, and only this combination allowed all Cys residues to form contacts with a partner  $<3.5$  Å away. Thus, this disulphide bonding pattern was most consistent with the experimental data for both domains, and was imposed (initially as NOE-style distance restraints) in subsequent calculations. The [1-3, 2-4, 5-6] pattern is that expected for an EGF-like domain.

*Hydrogen Bonds:* Non-exchanging amide groups involved in stable hydrogen bonds were identified in spectra of samples examined in 100 %  $D_2O$ . The corresponding hydrogen bond acceptors were determined by examining the initial structural ensemble, using the Insight II and HBPlus (McDonald *et al.*, 1994) programs, and hydrogen bond distance restraints were included in subsequent calculations. Further hydrogen bonds were identified in a similar manner in iterative calculations. Only 10 backbone hydrogen bonds in the antiparallel  $\beta$  sheets were used as restraints. Two distance restraints were used for each hydrogen bond, 1.7 - 2.3 Å from proton to acceptor, and 3.0 - 3.6 Å from donor nitrogen atom to acceptor.

### Structure Calculations

All the structure calculations were performed following standard protocols for *ab initio* simulated annealing from an extended chain using X-PLOR version 3.843 on a Silicon  
5 Graphics Origin 200 computer. The initial calculations used an initial temperature of 1000K, and 9000 steps of 5 fs in the restrained molecular dynamics stage. A soft-square potential was used for distance restraints. The SHAKE (Ryckaert *et al.*, 1977) algorithm was employed during molecular dynamics to maintain correct bond lengths. Refinement used a square well potential for restraints, and a final slow cooling of 30000 steps of 4 fs  
10 each from 2000K. A modified "parallhdg.pro" force-field parameter set was used, with modifications to parameters for Arg and Pro residues, and for hydrogen bonds (Polshakov *et al.*, 1997). Force constants were 50 kcal mol<sup>-1</sup> Å<sup>-2</sup> for all distance restraints including hydrogen bonds, and 200 kcal mol<sup>-1</sup> rad<sup>-2</sup> for dihedral restraints. The N-terminal sequence including the vector-encoded residues and (His)<sub>6</sub> tag was excluded from the structure  
15 calculations. All peptide bonds were constrained to be trans. NOE data for all 5 Pro residues showed strong H<sub>α(i-1)</sub>-ProH<sub>α</sub> crosspeaks, consistent with the trans peptide conformation.

Initial structures were calculated as described above to determine the disulphide bonding  
20 pattern. Then the calculation was repeated with identical NOE-derived distance and dihedral angle restraints, with the addition of 6 distance restraints (1.92-3.12 Å) representing the disulphide bridges. A new set of 50 structures was obtained, from which the best 20 structures were selected. The criteria used for selection were that the structures were below the median value of both total X-PLOR energy and rms NOE  
25 difference, and had no dihedral angle violations. The resulting structures had good geometry and between zero and two NOE violations > 0.5 Å. These structures were used to assign previously ambiguous NOEs and to determine the hydrogen bonds as described above.

30 The final structure calculation and refinement used an expanded restraint list including hydrogen bonds, additional dihedral restraints, stereoassignments of β-methylene and Leu δ signals, and more precisely calibrated ROE data (see Table 1). A set of 100 structures



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was obtained using this list, and 38 structures with 0-2 NOE violations  $> 0.5 \text{ \AA}$  and no dihedral angle violations  $> 5^\circ$  were accepted. These 38 structures were refined by the slow-cooling procedure described above, producing a final ensemble of 32 accepted structures with no NOE violations  $> 0.5 \text{ \AA}$  and no dihedral angle violations  $> 5^\circ$ . These selection criteria produced an ensemble of structures that extend to the end of the continuum of total potential energies in order to include structures having large scale correlated motions (Abseher *et al.*, 1998). Statistics for the final ensemble are given in Table 1. Coordinates for the 32 refined structures have been deposited in the Brookhaven Protein Data Bank (coordinates ID code 1cej; NMR restraints ID code r1cejmr).

Structures were analyzed during the calculation process using X-PLOR 3.8 (Nilges *et al.*, 1991), PROCHECK-NMR/AQUA (Laskowski *et al.*, 1996), and Insight II for quality of agreement with experimental data, precision, geometry, and energy. Models were aligned with Insight II and fitpdb, and displayed with Insight II, MOLSCRIPT (Kraulis, 1991), and GRASP (Nicholls *et al.*, 1991).

Table 1.

## A: RESTRAINTS SUMMARY

Number of conformers calculated: 100      Number of conformers accepted: 32

**Acceptance criteria:**No distance violation:  $> 0.5 \text{ \AA}$ No dihedral angle violation:  $> 5^\circ$ **NOE/ROE distance restraints:**

Intraresidue: 73

Sequential: 222

Medium range ( 2-4 ): 90

Long range (  $> 4$  ): 185

Total: 570

**Dihedral angle restraints:**

phi: 25

psi: 33

chi-1: 22

chi-2: 5

Total: 85

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Hydrogen bonds: 10 Disulphide bonds: 6

## B: STRUCTURE QUALITY

	<i>average</i>	<i>±/- s.d.</i>
5 <i>Total X-PLOR energy</i> (kcal mol <sup>-1</sup> )	168	20
<i>NOE X-PLOR energy</i> (kcal mol <sup>-1</sup> )	21	8
<i>rmsd NOE</i>	0.026	0.005
<i>rmsd dihedral angle</i>	0.236	0.095
10 <i>rmsd bond length</i>	0.0029	0.0002
<i>rmsd bond angle</i>	0.357	0.023
<i>rmsd improper</i>	0.266	0.018

**Backbone rmsd of structured region:** (69 residues)

15 Overall:	1.05	0.28
Domain-1:	0.81	0.32
Domain-2:	0.83	0.35

**Ramachandran plot quality (phi/psi angles):**

20 Most favoured	49.5 %
Additional allowed	42.1 %
Generously allowed	5.6 %
Disallowed	2.7 %

25 Monoclonal antibodies (mAbs)

Anti-MSP-1<sub>19</sub> monoclonal antibodies used in this study were : mouse IgG mAbs 1E1, 1E8, 2F10, 111.2, 111.4 2.2, 5.2, 7.5, 9C8, 12.8, 12.10, 12D11, 117.2, 8A12 (Holder *et al.*, 1985; McBride & Heidrich, 1987; Blackman *et al.*, 1987; Guevara Patiño *et al.*, 1997); and mouse IgM mAb 5B1 (Pirson & Perkins, 1985). Of these, mAbs 12.8, 12.10 and 5B1 are neutralising, inhibitory antibodies and 1E1, 2.2, 7.5, 9C8 and 111.4 are blocking antibodies. Some antibodies such as 111.2 are neither inhibitory nor blocking.

Construction of modified MSP-1<sub>19</sub> clones

The DNA coding the wild type MSP-1<sub>19</sub> domain of *Plasmodium falciparum* (T9-94/Wellcome strain) MSP-1 has been cloned in expression vector pGEX-3X to produce MSP-1<sub>19</sub> fused to the carboxy-terminus of the *Schistosoma japonicum* glutathione S-transferase (GST) in *Escherichia coli* (Burghaus & Holder, 1994). Site-directed mutagenesis of MSP-1<sub>19</sub> DNA sequence was done in either of two ways.

The first method was a modification of the method of Perrin & Gilliland (1990) to carry out polymerase chain reaction (PCR)-mediated site specific mutagenesis. DNA was amplified using the plasmid as a template together with one oligonucleotide to introduce the point mutation and a 5' primer from outside of the MSP-1<sub>19</sub> sequence. The amplified product was purified after electrophoresis on an agarose gel and used in a second amplification step together with a 3' primer from outside of the other end of the MSP-1<sub>19</sub> sequence and the plasmid as template. This second PCR product was digested with the restriction enzymes *Eco*R1 and *Bam*H1 and the product consisting of the modified MSP-1<sub>19</sub> coding sequence was inserted back into pGEX-3X and the products were used to transform DH5α cells.

The second method used the QuikChange™ Site-directed mutagenesis kit from Stratagene. Briefly, using the plasmid pGEX-MSP-1<sub>19</sub> as a template, two complementary synthetic oligonucleotide primers containing the desired point mutation were designed and were extended on the template by temperature cycling with the enzyme *Pfu* DNA Polymerase. This incorporation of the oligonucleotide primers results in the generation of a mutated plasmid containing staggered nicks in the DNA sequence. Following the temperature cycling, the product was treated with *Dpn*I endonuclease which will digest the methylated parental DNA template and leaves the mutation-containing newly synthesised DNA intact. The DNA incorporating the desired mutation was then transformed into *E. coli* strain DH5α (Life technologies) competent cells where the nicks will be repaired.

Clones were screened by analysis of restriction enzyme digests and by PCR screening of

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the insert gene. The DNA sequence of the selected mutant clones was confirmed using a PerkinElmer Applied Biosystems ABI 377 automatic sequencer according to the manufacturer's instructions.

5    Expression of the GST-MSP-1<sub>19</sub> fusion proteins

Expression of GST-MSP-1<sub>19</sub> was induced with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG; Melford Laboratories) for 1 hour in the *E. coli* strain TOPP 1 (Stratagene). The cells were then harvested by centrifugation and the cell pellet was  
10    resuspended in cell lysis buffer (50 mM Tris-HCl/1 mM EDTA pH 8.0 containing 0.2% (v/v) Nonidet P40 (NP40; BDH). Phenylmethylsulphonyl fluoride (PMSF; Sigma) in isopropanol was added to a final concentration of 1 mM. The cell suspension was sonicated, on ice, using VibraCell sonicator (Sonics & Materials) at 50% duty cycle for 3 min (six 30 sec pulses with 30 sec in between). The cell lysate was centrifuged at 65000 x  
15    g for 1 hour at 4°C. Supernatant containing soluble GST-fusion protein was applied to a glutathione-agarose column (Sigma) and the GST-fusion protein was eluted with 5 mM reduced glutathione. The eluted GST-fusion protein was dialysed extensively against phosphate buffered saline (PBS) at 4°C.

20    SDS-PAGE and Western Blotting

Proteins were analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). Samples were solubilised in SDS-PAGE buffer without reducing agents, then fractionated on a homogeneous 12.5% polyacrylamide gel. The pre-  
25    stained low range molecular mass markers (24-102kDa) from Bio-Rad were used as markers. When required, SDS-PAGE-fractionated polypeptides were either stained with Coomassie Brilliant Blue R-250 (CBB; Sigma) or electrophoretically transferred to Optitran BA-S 83 reinforced nitrocellulose (Schleicher & Schull, 0.2  $\mu$ m pore size) for analysis by western blotting. Blots were blocked with 5% BSA, 0.5% Tween 20 in PBS  
30    (PBS-T) for 1 h at room temperature, then washed in PBS-T. Blots were probed with first antibodies for 2 h at room temperature, washed 3 times in PBS-T, and then incubated in 1/1000 dilution of horse radish peroxidase (HRP)-conjugated sheep anti-mouse IgG (H+L)

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(ICN Immunobiologicals) or Goat anti-mouse IgM ( $\mu$  chain) (Sigma) for 1 h at room temperature. Blots were then washed 3 times in PBS-T and developed using Super Signal Substrate (Pierce) as HRP substrate for 1 min. Blots were then placed in plastic wrap and exposed to X-ray film (XB-200, X-ograph Imaging Systems). The films were processed with an Agfa Gevamat60 film processor (Agfa).

#### Analysis of antibody-antigen interaction using a BIAcore machine

GST-MSP-1<sub>19</sub> containing either the wild type or various modified sequences was used to coat a carboxymethyl dextran hydrogen sensor chip by the following methodology. The binding of the GST-MSP-1<sub>19</sub> was via amino groups using EDC/NHS chemistry. Immobilisation was done with the amine coupling kit (Pharmacia BIAcore). The CM dextran surface was activated with 50  $\mu$ l of 200 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 5 mM N-hydroxysuccinamide (NHS) for 10 min. GST-MSP-1<sub>19</sub> was then coupled to the BIAcore sensor surface using 50  $\mu$ l of a solution at 100  $\mu$ g ml<sup>-1</sup> in coating buffer (0.01M sodium acetate buffer, pH 3.5) for 10 min. Unreacted carboxyl groups were blocked by adding 50  $\mu$ l 1 M ethanolamine, pH 8.5 for 10 min. The cells were washed with two pulses of 20  $\mu$ l 10 mM glycine-HCl, pH 2.8 for 8 min in total to remove any non-covalently bound protein. The immobilisation procedure was carried out at a flow rate of 5  $\mu$ l min<sup>-1</sup>. Measurements were performed on the BIAcore 2000 instrument.

## RESULTS

### **Example 1 - Resonance assignments, NMR restraints and structure determination**

The assignments and restraints were obtained as described in Materials and Methods using a range of multidimensional heteronuclear experiments with <sup>13</sup>C/<sup>15</sup>N uniformly labelled protein. Sample spectra from 3D and 4D experiments showing NOE connections to the Lys35 backbone amide NH proton, resolved and unambiguously assigned using the <sup>13</sup>C chemical shift information, are shown in Figure 2. The distance, dihedral angle and hydrogen bond restraints used in the final set of structure calculations are summarized in

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Table 1. A total of 570 unambiguously assigned distance restraints, 85 dihedral angle restraints, and 10 hydrogen bonds were used in the final set. The assignments and restraint list shown in Table A have been submitted to the BioMagResBank database. Three disulphide bonds, with the (1-3, 2-4, 5-6) pattern for each domain were experimentally  
 5 determined from the NMR data in preliminary calculations as described in Materials and Methods, and these were also included in the final refinement. A final set of 32 models was calculated and refined using these restraints and these structures are shown in Figure 3 superimposed on the backbone of the representative structure  $S_{rep}$ . Table 1 shows that all 32 models have good geometry and are in good agreement with the experimental data  
 10 with no NOE violations  $> 0.5 \text{ \AA}$  and no dihedral angle violations  $> 5^\circ$ . The atomic rmsd value for the backbone atoms of the well-structured region (residues 15 - 64, 74 - 92) is  $1.05 \text{ \AA}$  (see Table 1). The local backbone rmsd is highest at the N-terminus (up to Cys12), in the loop Glu65 - Lys73, and following Cys92 at the C-terminus. The Ramachandran plot quality is typical of that found for other EGF structures (Doreleijers *et al.*, 1998).

## Description of the structure

### EGF-domains

Analysis of the final ensemble by PROCHECK-NMR indicated that each domain contains  
 20 a major stretch of antiparallel  $\beta$ -sheet containing the third and fourth Cys residues of each domain, as expected for an EGF-like fold, as well as an additional minor antiparallel  $\beta$ -sheet at the C-terminal end of domain-1, similar to some (but not all) EGF family members. These secondary structure features, together with the disulphide bonding patterns, can be seen in Figure 4. There is also a well-defined type II tight turn in domain-  
 25 1, with a hydrogen bond from Tyr 34NH proton to Leu31 carbonyl oxygen. The normally conserved EGF consensus Gly residue in the tight turn is replaced in domain-1 by a residue with a positive  $\phi$  angle (Asn33), while the conserved aromatic residue is present (Tyr34). There is a probable hydrogen bond between Leu 31NH proton to Asn15 carbonyl oxygen. Domain-2 contains two turns preceding the major  $\beta$ -sheet, (Asn53 - Cys56,  
 30 Asp57 - Ala60), and a final bend from Leu86 - Phe91 with a probable hydrogen bond from Asp57 NH proton to the carbonyl oxygen of Ile90 or Gly89. A surface-exposed loop from Pro81 to Pro85 replaces the tight turn, while the aromatic residue is not conserved.

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The large loop at the end of the major  $\beta$ -sheet (Glu65 - Lys73) is relatively disordered, and high mobility for the segment Gly68 - Gly71 was confirmed by backbone amide  $^{15}\text{N}\{^1\text{H}\}$  heteronuclear NOE measurements (Barbato *et al.*, 1992). The heteronuclear NOE values are dramatically reduced for residues in this region. At the N-terminus: the low NOE intensities correspond to increased mobility compared with the rest of the protein. The interdomain linker region from Pro45 to Pro47 is distinct from other EGF-like module pairs. The conformations of the disulphide bridges between Cys30 - Cys41 in domain-1, and the three Cys - Cys bonds in domain-2 are all left handed spirals (Richardson, 1981). Bridges between Cys30 - Cys41, Cys56 - Cys76, and Cys78 - Cys92 are particularly close to their equivalents in the blood coagulation factor Xa structure (1hcg). The conformations of the first two disulphide bridges in the relatively disordered N-terminal segment of domain-1 were not determined.

Figure 5 shows the backbone C, N,  $\text{C}_\alpha$  atom alignments of the two MSP-1 C-terminal fragment domains made with typical examples of EGF-like domains from several proteins, using the fitpdb program. Pairwise alignments showed that the two domains from MSP-1 are more similar to the factor Xa structure and its close relative from Clr, than to each other or to the other structures tested. The rmsd values for MSP-1 domains compared to factor Xa are comparable to those of the more distantly related structures fibrillin-1 and transforming growth factor- $\alpha$ .

The overall fold of each MSP-1 domain is thus similar to typical EGF family members, with the turns following the fifth Cys residue roughly equivalent, in spite of the divergence from the EGF consensus (C(5)xxG $\alpha$ ) where  $\alpha$  is a Phe or Tyr residue. Although some of the external loops are disordered, the scaffold is quite stable, as indicated by the non-exchangeable backbone amides (see above and in Protein Data Bank/BioMagResBank submission for details).

Unlike many EGF-like domains such as fibrillin-1, the MSP-1 C-terminal fragment lacks the conserved EGF  $\text{Ca}^{2+}$ -binding sequence and there was no evidence of  $\text{Ca}^{2+}$  binding to the MSP-1 C-terminal fragment. The 2D  $^1\text{H}$ -NOESY spectra were virtually identical in the absence or presence of 20 mM  $\text{CaCl}_2$ , indicating that any binding that might occur has,

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at most, only a small affect the overall structure.

#### *Domain interface and surface*

The most striking feature of the MSP-1 C-terminal fragment structure is the interface  
5 between the domains, which consists of several nonpolar amino acids (Phe19, Leu31, Leu32, Leu86, Phe87, Ile90 and Phe91) involved in hydrophobic interactions. These residues join the base of the major  $\beta$ -sheet and the tight turn in domain-1 with the final bend from residue 86 to 91 in domain-2. The domain interactions result in the domains forming a U-shaped structure which contrasts with structures observed for other pairs of  
10 EGF domains (Downing *et al.*, 1996; Brandstetter *et al.*, 1995). For example, in fibrillin-1, the interface between EGF domains 32 and 33 is largely formed by a shared  $\text{Ca}^{2+}$  ligation site (Downing *et al.*, 1996), and the overall structure resembles a rigid rod, with distant N- and C-termini. This contrasts with MSP-1 where the EGF-like domains are folded against each other so that their termini are relatively close together. A comparison of fibrillin-1 and MSP-1 EGF module pairs is shown in Figure 6. Although both termini of the MSP-1  
15 C-terminal fragment are somewhat disordered, NOE contacts were observed between nuclei in the two ends. The proximity of the C- and N-terminal positions may be significant, since it suggests that the proteolytic processing site that produces the C-terminal 96 amino acid fragment may be very close to the GPI membrane attachment site  
20 at or near residue 96. This proximity is consistent with the idea that a membrane-bound *Plasmodium* proteinase is responsible for secondary processing.

The electrostatic potential surface of the MSP-1 C-terminal fragment is shown in two views in Figure 7. The surface in Figure 7a is highly charged, especially in the protruding  
25 loop regions 23 - 27, 35 - 40 and 64 - 66. The surface in Figure 7b contains more neutral hydrophilic residues as well as a small hydrophobic patch from Pro85 - Phe87 near the center of the surface. In the future, such information could assist in understanding how these different surfaces may be involved in interactions with the rest of the MSP-1 precursor, the processing proteinase, other proteins on the merozoite surface, or unknown  
30 targets on the erythrocyte or parasite vacuolar membrane surfaces.



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### Primary sequence conservation

The residues involved in the hydrophobic domain interface in *P. falciparum* are also shown in Figure 1, together with corresponding residues in MSP-1 of the less virulent human malaria parasite, *P. vivax* (Del Portillo *et al.*, 1991; Gibson *et al.*, 1992). Extensive conservation of the interface residues (with conservative substitutions) suggests that *P. vivax* and perhaps other *Plasmodium* species as well, may have a similar U-shaped EGF module pair arrangement. Another feature of the *P. vivax* sequence, also seen in other *Plasmodium* species, is the single disulphide bond deficiency in the first EGF-like domain resulting from the absence of cysteine residues equivalent to the *P. falciparum* Cys12 and Cys28.

### *P. falciparum* dimorphic sites

Five dimorphic sites have been observed in the *P. falciparum* MSP-1<sub>19</sub> C-terminal fragment from different isolates (Qari *et al.*, 1998). Several observations can be made about the position of these sites on the MSP-1 structure. Two sites, Gln14/Glu14 and Lys61/Thr61, involve residues in relatively well-structured backbone regions, with surface-exposed hydrophilic or charged side-chains. A pair of adjacent sites, with the sequence variants Asn70 - Gly71/Ser70 - Arg71, occurs in the disordered loop of domain-2, within a segment (residues 68 - 71) that has been shown to be highly mobile. The region from Glu65 to Lys73 also appears to be the most variable region among different *Plasmodium* species (Daly *et al.*, 1992; Holder *et al.*, 1992). Finally, the fifth site has a substitution between hydrophobic residues (Leu86/Phe86). This partially-exposed side-chain is located at the hydrophobic domain interface, and the conservative substitution is consistent with a role in this interaction.

### Example 2 - Mutation and Monoclonal Antibody Binding studies

As a step towards understanding antibody interactions with the MSP-1 C-terminal fragment, the effect of engineered point mutations (within domain-1) on antibody binding has been studied. Amino acid substitutions were made that consisted of radical changes.

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These radical changes consisted of, for example, replacing an aliphatic residue with a charged polar residue, replacing a positively charged side chain with a negatively charged side chain, replacing an amino acid with a large side chain with an amino acid with a smaller or no side chain (glycine), replacing a polar amino acid with a charged polar amino acid, replacing a polar amino acid with an aromatic amino acid, replacing a large aromatic amino acid with an amino acid with a small side chain, and replacing cysteine residues that are involved in disulphide bonds.

Four individual amino acid substitutions shown in Figure 8, each completely abolish binding of one or more mAbs to the mutant fragment, as detected by Western blotting. The Glu26 mutation, shown in cyan, is closest to the N-terminal proteolytic processing site (magenta) at Asn1, and is the only one of this group of mutations that affects binding of a processing-inhibitory antibody, i.e. one that is capable of preventing both proteolytic processing of the MSP-1 precursor and erythrocyte invasion *in vitro*. The other three mutations abolish binding of blocking antibodies that bind to the native C-terminal fragment and interfere with the binding of processing-inhibitory antibodies.

Additional mutations were made based on the immunochemical analyses and the tertiary structure of the molecule, and the binding of the mAbs was assessed by western blotting and BIAcore analysis. The results are summarised in Table 2. The results of the binding of selected mAbs to the modified proteins as detected by Western blotting are shown in Figure 9, and by BIAcore analysis in Figure 10. Some individual amino acid changes have no effect on the binding of any of the mAbs tested (for example Leu22 to Arg). Other substitutions affect the binding of one or more mAbs.

Of particular interest are those changes that prevent the binding of blocking antibodies but have no effect on the binding of the inhibitory antibodies. For example, replacement of Asn15 by Arg prevents the binding of mAb 7.5, replacement of Glu27 by Tyr prevents the binding of mAb 2.2, replacement of Leu31 by Arg prevents the binding of mAb 1E1, replacement of Tyr34 by Ser prevents the binding of mAb 7.5, and replacement of Glu43 by Leu prevents the binding of mAb 111.4.

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Several combinations of substitutions that prevent the binding of blocking antibodies but do not affect the binding of inhibitory antibodies were made in single proteins (Table 2 and Figure 11). In the first Glu27→Tyr, Leu31→Arg and Glu43→Leu were combined, in the second Glu27→Tyr, Leu31→Arg, Tyr34→Ser, and Glu43→Leu were combined, and the third Asn15→Arg, Glu27→Tyr, Leu31→Arg and Glu43→Leu were combined. None of these modified proteins bound any of the blocking antibodies but continued to bind the inhibitory antibodies. We propose that the mutant proteins will induce a polyclonal response that is more inhibitory than that induced by the wild type protein.

The modified recombinant proteins will also be used to affinity select antibodies from pooled serum from individuals exposed to malaria. We hypothesise that the modified proteins will select less blocking antibody than the wild type protein and that therefore these selected antibodies will be more effective in inhibiting parasite invasion *in vitro* and secondary processing.

In the first EGF-like domain of MSP-1 from the rodent, primate and *P. vivax* malaria parasites, cysteines 2 and 4 are not present. We have replaced this cysteine pair (Cys12 and Cys28) in the *P. falciparum* protein. This does not appear to have any effect on the binding of any of the inhibitory antibodies, but does abolish the binding of the blocking antibody mAb 2.2. We propose that one reason why the proteins from these other malaria parasites are more immunogenic is that T cell recognition is more effective or that processing by antigen processing cells proceeds by a different degradation pathway that drives the fine specificity of the antibody response in a more productive direction (see for example Egan *et al.*, 1997). Removal of the cysteine pair may improve the immunogenicity of the modified protein and this will be assessed by measuring the level of antibodies induced by the *P. falciparum* protein without the two cysteines with the level of antibodies induced by the wild type protein.

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Table 2 - The location of amino acid sequence changes and their effect on the binding of monoclonal antibodies

Position	Amino acid		Monoclonal Antibody binding														
	Wild type	mutant	12.8	12.10	5B1	1E1	2.2	7.5	111.4	111.2	9C8	2F10	12D11	117.2	5.2	1E8	8A12
6	Gln	Ile	++	++	++	++	+	+	++	++	++	++	++	++	++	++	++
14	Gln	Gly	++	++	++	++	++	+	+	++	++	++	++	+	++	++	++
14	Gln	Arg	++	++	++	++	++	+	+	++	+	++	++	+	++	++	++
15	Asn	Arg	++	++	++	++	-	-	+	++	++	++	++	+	++	+	++
20	Arg	Glu	+	++	+	++	+	+	++	+	++	++	++	+	++	++	++
22	Leu	Arg	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
24	Glu	Lys	+	++	++	++	++	++	++	++	+	++	++	++	++	+	++
25	Arg	Gly	++	++	++	++	++	++	++	++	++	++	++	++	++	+	++
26	Glu	Ile	-	++	+	++	-	-	++	++	++	++	++	++	+	+	+
27	Glu	Tyr	++	++	++	++	++	+	++	++	++	++	++	++	++	++	++
29	Lys	Ser	+	++	++	++	++	++	++	++	++	++	++	++	++	++	+
31	Leu	Arg	+	++	++	++	++	++	++	-	+	++	++	++	++	++	++
32	Leu	Arg	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
33	Asn	Ile	++	++	++	++	++	++	++	+	++	++	++	++	++	++	++
34	Tyr	Ser	++	++	++	+	+	+	+	++	+	++	+	+	++	++	++
34	Tyr	Ile	++	++	++	+	+	+	++	++	++	++	++	++	++	++	++
35	Lys	Ile	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
36	Gln	Gly	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
37	Glu	Ile	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
39	Asp	Thr	++	++	++	++	++	++	++	++	++	++	++	++	++	+	++
40	Lys	Ile	+	++	+	+	++	++	+	+	+	+	+	+	++	++	+
43	Glu	Leu	++	++	++	+	++	+	-	++	++	+	++	++	++	++	++
48	Thr	Lys	++	++	++	++	++	++	++	++	++	-	+	+	++	++	++
53	Asn	Arg	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
80	Lys	Ile	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Wild type			++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

++ = strong binding, + = binding, - = no binding

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Table 2 (cont.)

Position Combinations	Amino acid		Monoclonal antibody binding														
	Wildtype	Mutant	12.8	12.10	5B1	1E1	2.2	7.5	111.4	111.2	9C8	2F10	12D11	117.2	5.2	1E8	8A12
12+28	Cys	Ile	++	++	++	++	-	++	++	+	+	++	++	+	-	++	++
	Cys	Trp	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Cys	Ala	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
14+18	Cys	Phe	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Gln	Gly	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Cys	Tyr	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
14+18	Gln	Arg	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Cys	Tyr	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Tyr	Ser	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
34+39	Asp	Asn	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Leu	Leu	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Thr	Ile	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
43+48	Glu	Leu	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Thr	Asn	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Pro	Ser	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
47+48	Thr	Lys	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Glu	Tyr	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Leu	Arg	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
27+31+43	Glu	Leu	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Thr	Asn	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Pro	Ser	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
27+31+34+43	Glu	Tyr	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Leu	Arg	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Tyr	Ser	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
15+27+31+43	Glu	Leu	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Asn	Arg	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Glu	Tyr	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
12+15+27+31+43	Leu	Arg	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Glu	Tyr	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Leu	Arg	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
12+15+27+31+43	Glu	Leu	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Cys	Ile	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Asn	Arg	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
12+15+27+31+43	Glu	Tyr	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Leu	Arg	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
	Glu	Leu	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++

++ = strong binding, + = binding, - = no binding

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TABLE A

```

#
# 13-10-98
# merozoite surface protein-1 (MSP-1) Plasmodium falciparum (C-terminal fragment)
# Reference: 1H : DSS=0.000 dioxane=3.755 (internal)
# 15N: indirect
# 13C: indirect
# 25C pH 6.5 50mM NaPO4 100mM NaCl 90%H2O/10%D2O
# --FORMAT--
# BioMagResBank
#
# The original sequence entered was:
#
NISQHCQVKKQCPQNSGCFRHLDEREECKLLNYKQEGDKCVENPNPTCNENNGGCDADAKCTEEDSGSNGK
KITCECTKPD SYPLFDGIFC SSSN
#
# Expressed in NMR-STAR, this sequence is:

_Mol_residue_sequence
;
NISQHCQVKKQCPQNSGCFR
HLDEREECKLLNYKQEGDK
CVENPNPTCNENNGGCDADA
KCTEEDSGSNGKKITCECTK
PDSYPLFDGIFC SSSN
;
loop_
  _Residue_seq_code
  _Residue_author_seq_code
  _Residue_label

1 @ ASN      2 @ ILE      3 @ SER      4 @ GLN      5 @ HIS
6 @ GLN      7 @ CYS      8 @ VAL      9 @ LYS     10 @ LYS
11 @ GLN     12 @ CYS     13 @ PRO     14 @ GLN     15 @ ASN
16 @ SER     17 @ GLY     18 @ CYS     19 @ PHE     20 @ ARG
21 @ HIS     22 @ LEU     23 @ ASP     24 @ GLU     25 @ ARG
26 @ GLU     27 @ GLU     28 @ CYS     29 @ LYS     30 @ CYS
31 @ LEU     32 @ LEU     33 @ ASN     34 @ TYR     35 @ LYS
36 @ GLN     37 @ GLU     38 @ GLY     39 @ ASP     40 @ LYS
41 @ CYS     42 @ VAL     43 @ GLU     44 @ ASN     45 @ PRO
46 @ ASN     47 @ PRO     48 @ THR     49 @ CYS     50 @ ASN
51 @ GLU     52 @ ASN     53 @ ASN     54 @ GLY     55 @ GLY
56 @ CYS     57 @ ASP     58 @ ALA     59 @ ASP     60 @ ALA
61 @ LYS     62 @ CYS     63 @ THR     64 @ GLU     65 @ GLU
66 @ ASP     67 @ SER     68 @ GLY     69 @ SER     70 @ ASN
71 @ GLY     72 @ LYS     73 @ LYS     74 @ ILE     75 @ THR
76 @ CYS     77 @ GLU     78 @ CYS     79 @ THR     80 @ LYS
81 @ PRO     82 @ ASP     83 @ SER     84 @ TYR     85 @ PRO
86 @ LEU     87 @ PHE     88 @ ASP     89 @ GLY     90 @ ILE
91 @ PHE     92 @ CYS     93 @ SER     94 @ SER     95 @ SER
96 @ ASN

stop_

```

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```
#####
#   Chemical Shift Ambiguity Code Definitions   #
#   Codes   Definition                         #
#   1       Unique                           #
#   2       Ambiguity of geminal atoms or geminal methyl #
#           proton groups                     #
#   3       Aromatic atoms on opposite sides of the ring#
#           (e.g. Tyr HE1 and HE2 protons)   #
#   4       Intraresidue ambiguities (e.g. Lys HG and #
#           HD protons)                      #
#   5       Interresidue ambiguities (Lys 12 vs. Lys 27) #
#   9       Ambiguous, specific ambiguity not defined #
#   #####
```

## # INSTRUCTIONS

- # 1) Replace the @-signs with appropriate values.  
 # 2) Text comments concerning the assignments can be supplied in the full deposition.  
 # 3) Feel free to add or delete rows to the table as needed.  
 # The row numbers (\_Atom\_shift\_assign\_ID values) will be re-assigned to sequential values by BMRB

# # The atom table chosen for this sequence is:

```
loop_
  _Atom_shift_assign_ID
  _Residue_seq_code
  _Residue_label
  _Atom_name
  _Atom_type
  _Chem_shift_value
  _Chem_shift_value_error
  _Chem_shift_ambiguity_code
```

#

Table A1. Supplementary: <sup>1</sup>H, <sup>13</sup>C and <sup>15</sup>N chemical shift assignments of MSP-1 C-terminal fragment

Atom shift assign	Residue Seq no.	Residue Name	Atom Name	Atom Type	Shift/ ppm	Error/ ppm	Ambiguity- Code
1	1	ASN	H	H	8.29	0.02	1
2	1	ASN	HA	H	4.60	0.02	1
3	1	ASN	HB2	H	2.86	0.02	2
4	1	ASN	HB3	H	2.75	0.02	2
5	1	ASN	HD21	H	?	?	?
6	1	ASN	HD22	H	?	?	?
7	1	ASN	C	C	?	?	?
8	1	ASN	CA	C	55.5	0.6	1
9	1	ASN	CB	C	40.9	0.6	1
11	1	ASN	N	N	125.8	0.3	1
12	1	ASN	ND2	N	?	?	?
13	2	ILE	H	H	8.29	0.02	1

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14	2	ILE	HA	H	4.25	0.02	1
15	2	ILE	HB	H	1.97	0.02	1
16	2	ILE	HG12	H	1.39	0.02	2
17	2	ILE	HG13	H	1.19	0.02	2
18	2	ILE	HG2	H	0.92	0.02	1
19	2	ILE	HD1	H	0.81	0.02	1
20	2	ILE	C	C	173.8	0.6	1
21	2	ILE	CA	C	62.2	0.6	1
22	2	ILE	CB	C	38.7	0.6	1
23	2	ILE	CG1	C	27.5	0.6	1
24	2	ILE	CG2	C	18.2	0.6	1
25	2	ILE	CD1	C	13.7	0.6	1
26	2	ILE	N	N	121.1	0.3	1
27	3	SER	H	H	8.47	0.02	1
28	3	SER	HA	H	4.20	0.02	1
29	3	SER	HB2	H	3.90	0.02	1
30	3	SER	HB3	H	3.90	0.02	1
32	3	SER	C	C	?	?	?
33	3	SER	CA	C	60.9	0.6	1
34	3	SER	CB	C	63.3	0.6	1
35	3	SER	N	N	119.3	0.3	1
36	4	GLN	H	H	8.32	0.02	1
37	4	GLN	HA	H	4.02	0.02	1
38	4	GLN	HB2	H	1.88	0.02	1
39	4	GLN	HB3	H	1.88	0.02	1
40	4	GLN	HG2	H	1.75	0.02	1
41	4	GLN	HG3	H	1.75	0.02	1
42	4	GLN	HE21	H	?	?	?
43	4	GLN	HE22	H	?	?	?
44	4	GLN	C	C	?	?	?
45	4	GLN	CA	C	57.7	0.6	1
46	4	GLN	CB	C	27.9	0.6	1
47	4	GLN	CG	C	32.7	0.6	1
49	4	GLN	N	N	121.6	0.3	1
50	4	GLN	NE2	N	?	?	?
51	5	HIS	H	H	7.76	0.02	9
52	5	HIS	HA	H	5.09	0.02	1
53	5	HIS	HB2	H	2.70	0.02	1
54	5	HIS	HB3	H	2.70	0.02	1
56	5	HIS	HD2	H	6.87	0.02	1
57	5	HIS	HE1	H	7.92	0.02	1
59	5	HIS	C	C	175.7	0.6	1
60	5	HIS	CA	C	54.8	0.6	1
61	5	HIS	CB	C	29.2	0.6	1
65	5	HIS	N	N	113.6	0.3	9
68	6	GLN	H	H	7.42	0.02	9
69	6	GLN	HA	H	4.43	0.02	1
70	6	GLN	HB2	H	2.05	0.02	1
71	6	GLN	HB3	H	2.05	0.02	1
72	6	GLN	HG2	H	2.42	0.02	1
73	6	GLN	HG3	H	2.42	0.02	1
74	6	GLN	HE21	H	7.59	0.02	5
75	6	GLN	HE22	H	6.92	0.02	5
76	6	GLN	C	C	175.7	0.6	1
77	6	GLN	CA	C	55.1	0.6	1
78	6	GLN	CB	C	28.8	0.6	1
79	6	GLN	CG	C	33.8	0.6	1
81	6	GLN	N	N	122.5	0.3	9



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82	6	GLN	NE2	N	112.6	0.3	5
83	7	CYS	H	H	9.18	0.02	1
84	7	CYS	HA	H	4.09	0.02	1
85	7	CYS	HB2	H	3.31	0.02	2
86	7	CYS	HB3	H	3.11	0.02	2
88	7	CYS	C	C	174.4	0.6	1
89	7	CYS	CA	C	56.6	0.6	1
90	7	CYS	CB	C	42.3	0.6	1
91	7	CYS	N	N	124.5	0.3	1
92	8	VAL	H	H	10.42	0.02	1
93	8	VAL	HA	H	4.33	0.02	1
94	8	VAL	HB	H	2.15	0.02	1
95	8	VAL	HG1	H	0.84	0.02	2
96	8	VAL	HG2	H	0.82	0.02	2
97	8	VAL	C	C	176.6	0.6	1
98	8	VAL	CA	C	62.5	0.6	1
99	8	VAL	CB	C	34.4	0.6	1
100	8	VAL	CG1	C	21.5	0.6	2
101	8	VAL	CG2	C	19.7	0.6	2
102	8	VAL	N	N	119.1	0.3	1
103	9	LYS	H	H	9.42	0.02	1
104	9	LYS	HA	H	4.51	0.02	1
105	9	LYS	HB2	H	1.81	0.02	4
106	9	LYS	HB3	H	1.81	0.02	4
107	9	LYS	HG2	H	1.41	0.02	4
108	9	LYS	HG3	H	1.41	0.02	4
109	9	LYS	HD2	H	?	?	?
110	9	LYS	HD3	H	?	?	?
111	9	LYS	HE2	H	3.33	0.02	1
112	9	LYS	HE3	H	3.33	0.02	1
114	9	LYS	C	C	?	?	?
115	9	LYS	CA	C	57.7	0.6	1
116	9	LYS	CB	C	?	?	?
117	9	LYS	CG	C	?	?	?
118	9	LYS	CD	C	?	?	?
119	9	LYS	CE	C	?	?	?
120	9	LYS	N	N	124.1	0.03	1
122	10	LYS	H	H	8.94	0.02	1
123	10	LYS	HA	H	4.06	0.02	1
124	10	LYS	HB2	H	1.86	0.02	1
125	10	LYS	HB3	H	1.86	0.02	1
126	10	LYS	HG2	H	1.29	0.02	1
127	10	LYS	HG3	H	1.29	0.02	1
128	10	LYS	HD2	H	1.70	0.02	2
129	10	LYS	HD3	H	1.59	0.02	2
130	10	LYS	HE2	H	3.04	0.02	1
131	10	LYS	HE3	H	3.04	0.02	1
133	10	LYS	C	C	?	?	?
134	10	LYS	CA	C	57.1	0.6	1
135	10	LYS	CB	C	34.3	0.6	1
136	10	LYS	CG	C	25.6	0.6	1
137	10	LYS	CD	C	29.6	0.6	1
138	10	LYS	CE	C	42.4	0.6	1
139	10	LYS	N	N	122.4	0.3	1
141	11	GLN	H	H	?	?	?
142	11	GLN	HA	H	4.47	0.02	1
143	11	GLN	HB2	H	2.03	0.02	2
144	11	GLN	HB3	H	1.89	0.02	2

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145	11	GLN	HG2	H	2.28	0.02	1
146	11	GLN	HG3	H	2.28	0.02	1
147	11	GLN	HE21	H	7.45	0.02	2
148	11	GLN	HE22	H	6.84	0.02	2
149	11	GLN	C	C	?	?	?
150	11	GLN	CA	C	54.4	0.6	1
151	11	GLN	CB	C	28.7	0.6	1
152	11	GLN	CG	C	33.8	0.6	1
154	11	GLN	N	N	?	?	?
155	11	GLN	NE2	N	112.9	0.3	1
156	12	CYS	H	H	?	?	?
157	12	CYS	HA	H	5.09	0.02	1
158	12	CYS	HB2	H	3.49	0.02	2
159	12	CYS	HB3	H	2.34	0.02	2
161	12	CYS	C	C	?	?	?
162	12	CYS	CA	C	52.4	0.6	1
163	12	CYS	CB	C	37.2	0.6	1
164	12	CYS	N	N	?	?	?
165	13	PRO	HA	H	4.55	0.02	1
166	13	PRO	HB2	H	2.45	0.02	1
167	13	PRO	HB3	H	1.94	0.02	1
168	13	PRO	HG2	H	1.73	0.02	2
169	13	PRO	HG3	H	2.04	0.02	2
170	13	PRO	HD2	H	3.43	0.02	2
171	13	PRO	HD3	H	3.80	0.02	2
172	13	PRO	C	C	176.4	0.6	1
173	13	PRO	CA	C	62.6	0.6	1
174	13	PRO	CB	C	33.0	0.6	1
175	13	PRO	CG	C	27.5	0.6	1
176	13	PRO	CD	C	50.6	0.6	1
178	14	GLN	H	H	8.48	0.02	1
179	14	GLN	HA	H	4.01	0.02	1
180	14	GLN	HB2	H	1.94	0.02	1
181	14	GLN	HB3	H	1.94	0.02	1
182	14	GLN	HG2	H	2.42	0.02	1
183	14	GLN	HG3	H	2.42	0.02	1
184	14	GLN	HE21	H	7.59	0.02	5
185	14	GLN	HE22	H	6.92	0.02	5
186	14	GLN	C	C	176.6	0.6	1
187	14	GLN	CA	C	57.4	0.6	1
188	14	GLN	CB	C	28.6	0.6	1
189	14	GLN	CG	C	33.8	0.6	1
191	14	GLN	N	N	120.5	0.3	1
192	14	GLN	NE2	N	112.6	0.3	5
193	15	ASN	H	H	8.93	0.02	1
194	15	ASN	HA	H	3.77	0.02	1
195	15	ASN	HB2	H	2.58	0.02	1
196	15	ASN	HB3	H	1.09	0.02	1
197	15	ASN	HD21	H	6.97	0.02	1
198	15	ASN	HD22	H	7.12	0.02	1
199	15	ASN	C	C	171.9	0.6	1
200	15	ASN	CA	C	54.6	0.6	1
201	15	ASN	CB	C	36.3	0.6	1
203	15	ASN	N	N	115.8	0.3	1
204	15	ASN	ND2	N	115.4	0.3	1
205	16	SER	H	H	7.34	0.02	1
206	16	SER	HA	H	4.93	0.02	1
207	16	SER	HB2	H	3.62	0.02	2

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208	16	SER	HB3	H	3.52	0.02	2
210	16	SER	C	C	173.5	0.6	1
211	16	SER	CA	C	57.5	0.6	1
212	16	SER	CB	C	67.9	0.6	1
213	16	SER	N	N	109.9	0.3	1
214	17	GLY	H	H	8.92	0.02	1
215	17	GLY	HA2	H	3.83	0.02	1
216	17	GLY	HA3	H	2.06	0.02	1
217	17	GLY	C	C	?	?	?
218	17	GLY	CA	C	42.6	0.6	1
219	17	GLY	N	N	108.5	0.3	1
220	18	CYS	H	H	7.01	0.02	1
221	18	CYS	HA	H	5.63	0.02	1
222	18	CYS	HB2	H	3.01	0.02	1
223	18	CYS	HB3	H	3.01	0.02	1
225	18	CYS	C	C	172.5	0.6	1
226	18	CYS	CA	C	55.8	0.6	1
227	18	CYS	CB	C	43.2	0.6	1
228	18	CYS	N	N	120.5	0.3	1
229	19	PHE	H	H	9.12	0.02	1
230	19	PHE	HA	H	4.43	0.02	1
231	19	PHE	HB2	H	1.70	0.02	2
232	19	PHE	HB3	H	0.62	0.02	2
233	19	PHE	HD1	H	6.12	0.02	1
234	19	PHE	HD2	H	6.12	0.02	1
235	19	PHE	HE1	H	6.30	0.02	1
236	19	PHE	HE2	H	6.30	0.02	1
237	19	PHE	HZ	H	6.37	0.02	1
238	19	PHE	C	C	172.3	0.6	1
239	19	PHE	CA	C	57.0	0.6	1
240	19	PHE	CB	C	41.7	0.6	1
247	19	PHE	N	N	132.0	0.6	1
248	20	ARG	H	H	7.76	0.02	1
249	20	ARG	HA	H	4.83	0.02	1
250	20	ARG	HB2	H	1.26	0.02	2
251	20	ARG	HB3	H	0.99	0.02	2
252	20	ARG	HG2	H	1.59	0.02	2
253	20	ARG	HG3	H	1.42	0.02	2
254	20	ARG	HD2	H	3.35	0.02	2
255	20	ARG	HD3	H	3.10	0.02	2
256	20	ARG	HE	H	7.18	0.02	1
257	20	ARG	HH11	H	6.23	0.02	5
258	20	ARG	HH12	H	6.23	0.02	5
259	20	ARG	HH21	H	6.23	0.02	5
260	20	ARG	HH22	H	6.23	0.02	5
261	20	ARG	C	C	174.7	0.6	1
262	20	ARG	CA	C	54.3	0.6	1
263	20	ARG	CB	C	32.3	0.6	1
264	20	ARG	CG	C	28.0	0.6	1
265	20	ARG	CD	C	44.1	0.6	1
267	20	ARG	N	N	129.4	0.3	1
268	20	ARG	NE	N	85.0	0.3	1
269	20	ARG	NH1	N	70.1	0.3	5
270	20	ARG	NH2	N	70.1	0.3	5
271	21	HIS	H	H	9.30	0.02	1
272	21	HIS	HA	H	4.50	0.02	1
273	21	HIS	HB2	H	3.52	0.02	2
274	21	HIS	HB3	H	3.44	0.02	2

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276	21	HIS	HD2	H	7.02	0.02	1
277	21	HIS	HE1	H	8.44	0.02	1
279	21	HIS	C	C	177.6	0.6	1
280	21	HIS	CA	C	56.4	0.6	1
281	21	HIS	CB	C	32.2	0.6	1
285	21	HIS	N	N	125.9	0.3	1
288	22	LEU	H	H	9.30	0.02	1
289	22	LEU	HA	H	4.11	0.02	1
290	22	LEU	HB2	H	1.87	0.02	1
291	22	LEU	HB3	H	1.65	0.02	1
292	22	LEU	HG	H	1.87	0.02	1
293	22	LEU	HD1	H	0.77	0.02	2
294	22	LEU	HD2	H	0.98	0.02	2
295	22	LEU	C	C	177.7	0.6	1
296	22	LEU	CA	C	57.7	0.6	1
297	22	LEU	CB	C	40.8	0.6	1
298	22	LEU	CG	C	27.6	0.6	1
299	22	LEU	CD1	C	22.6	0.6	2
300	22	LEU	CD2	C	25.3	0.6	2
301	22	LEU	N	N	122.0	0.3	1
302	23	ASP	H	H	7.86	0.02	1
303	23	ASP	HA	H	4.52	0.02	1
304	23	ASP	HB2	H	3.10	0.02	1
305	23	ASP	HB3	H	2.53	0.02	1
306	23	ASP	C	C	176.8	0.6	1
307	23	ASP	CA	C	53.6	0.6	1
308	23	ASP	CB	C	39.7	0.6	1
310	23	ASP	N	N	116.9	0.3	1
311	24	GLU	H	H	8.01	0.02	1
312	24	GLU	HA	H	3.63	0.02	1
313	24	GLU	HB2	H	2.57	0.02	2
314	24	GLU	HB3	H	2.17	0.02	2
315	24	GLU	HG2	H	2.14	0.02	1
316	24	GLU	HG3	H	2.14	0.02	1
317	24	GLU	C	C	176.1	0.6	1
318	24	GLU	CA	C	59.4	0.6	1
319	24	GLU	CB	C	27.5	0.6	1
320	24	GLU	CG	C	37.0	0.6	1
322	24	GLU	N	N	110.3	0.3	1
323	25	ARG	H	H	8.04	0.02	1
324	25	ARG	HA	H	4.24	0.02	1
325	25	ARG	HB2	H	1.84	0.02	2
326	25	ARG	HB3	H	1.75	0.02	2
327	25	ARG	HG2	H	1.56	0.02	1
328	25	ARG	HG3	H	1.56	0.02	1
329	25	ARG	HD2	H	3.16	0.02	1
330	25	ARG	HD3	H	3.16	0.02	1
331	25	ARG	HE	H	8.01	0.02	1
332	25	ARG	HH11	H	6.71	0.02	5
333	25	ARG	HH12	H	6.71	0.02	5
334	25	ARG	HH21	H	6.71	0.02	5
335	25	ARG	HH22	H	6.71	0.02	5
336	25	ARG	C	C	175.6	0.6	1
337	25	ARG	CA	C	57.8	0.6	1
338	25	ARG	CB	C	31.0	0.6	1
339	25	ARG	CG	C	28.3	0.6	1
340	25	ARG	CD	C	43.5	0.6	1
342	25	ARG	N	N	121.9	0.3	1

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343	25	ARG	NE	N	85.8	0.3	1
344	25	ARG	NH1	N	70.3	0.3	5
345	25	ARG	NH2	N	70.3	0.3	5
346	26	GLU	H	H	8.69	0.02	1
347	26	GLU	HA	H	5.40	0.02	1
348	26	GLU	HB2	H	1.90	0.02	1
349	26	GLU	HB3	H	1.90	0.02	1
350	26	GLU	HG2	H	2.63	0.02	2
351	26	GLU	HG3	H	2.09	0.02	2
352	26	GLU	C	C	173.6	0.6	1
353	26	GLU	CA	C	55.6	0.6	1
354	26	GLU	CB	C	37.9	0.6	1
355	26	GLU	CG	C	31.5	0.6	1
357	26	GLU	N	N	124.3	0.3	1
358	27	GLU	H	H	9.07	0.02	1
359	27	GLU	HA	H	4.84	0.02	1
360	27	GLU	HB2	H	2.15	0.02	2
361	27	GLU	HB3	H	2.27	0.02	2
362	27	GLU	HG2	H	2.48	0.02	2
363	27	GLU	HG3	H	2.32	0.02	2
364	27	GLU	C	C	174.5	0.6	1
365	27	GLU	CA	C	54.6	0.6	1
366	27	GLU	CB	C	34.8	0.6	1
367	27	GLU	CG	C	36.5	0.6	1
369	27	GLU	N	N	123.9	0.3	1
370	28	CYS	H	H	8.88	0.02	1
371	28	CYS	HA	H	5.65	0.02	1
372	28	CYS	HB2	H	3.00	0.02	2
373	28	CYS	HB3	H	2.81	0.02	2
375	28	CYS	C	C	175.5	0.6	1
376	28	CYS	CA	C	53.0	0.6	1
377	28	CYS	CB	C	40.9	0.6	1
378	28	CYS	N	N	122.0	0.3	1
379	29	LYS	H	H	8.76	0.02	1
380	29	LYS	HA	H	4.56	0.02	1
381	29	LYS	HB2	H	1.40	0.02	1
382	29	LYS	HB3	H	1.40	0.02	1
383	29	LYS	HG2	H	1.38	0.02	1
384	29	LYS	HG3	H	1.38	0.02	1
385	29	LYS	HD2	H	1.13	0.02	2
386	29	LYS	HD3	H	0.92	0.02	2
387	29	LYS	HE2	H	2.86	0.02	1
388	29	LYS	HE3	H	2.86	0.02	1
389	29	LYS	HZ	H	7.23	0.02	5
390	29	LYS	C	C	174.6	0.6	1
391	29	LYS	CA	C	56.4	0.6	1
392	29	LYS	CB	C	38.4	0.6	1
393	29	LYS	CG	C	25.8	0.6	1
394	29	LYS	CD	C	29.6	0.6	1
395	29	LYS	CE	C	42.4	0.6	1
396	29	LYS	N	N	124.0	0.3	1
397	29	LYS	NZ	N	33.0	0.3	5
398	30	CYS	H	H	8.78	0.02	1
399	30	CYS	HA	H	4.65	0.02	1
400	30	CYS	HB2	H	2.49	0.02	1
401	30	CYS	HB3	H	3.01	0.02	1
403	30	CYS	C	C	173.3	0.6	1
404	30	CYS	CA	C	54.6	0.6	1

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405	30	CYS	CB	C	35.9	0.6	1
406	30	CYS	N	N	121.7	0.3	1
407	31	LEU	H	H	7.81	0.02	1
408	31	LEU	HA	H	4.23	0.02	1
409	31	LEU	HB2	H	1.39	0.02	1
410	31	LEU	HB3	H	1.73	0.02	1
411	31	LEU	HG	H	0.94	0.02	1
412	31	LEU	HD1	H	0.68	0.02	1
413	31	LEU	HD2	H	0.77	0.02	1
414	31	LEU	C	C	176.2	0.6	1
415	31	LEU	CA	C	54.8	0.6	1
416	31	LEU	CB	C	42.5	0.6	1
417	31	LEU	CG	C	27.1	0.6	1
418	31	LEU	CD1	C	26.0	0.6	1
419	31	LEU	CD2	C	21.9	0.6	1
420	31	LEU	N	N	119.2	0.3	1
421	32	LEU	H	H	8.91	0.02	1
422	32	LEU	HA	H	4.26	0.02	1
423	32	LEU	HB2	H	1.75	0.02	1
424	32	LEU	HB3	H	1.32	0.02	1
425	32	LEU	HG	H	1.77	0.02	1
426	32	LEU	HD1	H	0.96	0.02	1
427	32	LEU	HD2	H	0.77	0.02	1
428	32	LEU	C	C	179.0	0.6	1
429	32	LEU	CA	C	56.4	0.6	1
430	32	LEU	CB	C	42.1	0.6	1
431	32	LEU	CG	C	26.9	0.6	1
432	32	LEU	CD1	C	26.0	0.6	1
433	32	LEU	CD2	C	23.4	0.6	1
434	32	LEU	N	N	118.4	0.3	1
435	33	ASN	H	H	8.84	0.02	1
436	33	ASN	HA	H	3.90	0.02	1
437	33	ASN	HB2	H	3.26	0.02	1
438	33	ASN	HB3	H	2.88	0.02	1
439	33	ASN	HD21	H	6.86	0.02	1
440	33	ASN	HD22	H	7.25	0.02	1
441	33	ASN	C	C	173.8	0.6	1
442	33	ASN	CA	C	56.9	0.6	1
443	33	ASN	CB	C	37.3	0.6	1
445	33	ASN	N	N	110.8	0.3	1
446	33	ASN	ND2	N	112.0	0.3	1
447	34	TYR	H	H	8.61	0.02	1
448	34	TYR	HA	H	5.02	0.02	1
449	34	TYR	HB2	H	3.35	0.02	1
450	34	TYR	HB3	H	2.52	0.02	1
451	34	TYR	HD1	H	6.74	0.02	1
452	34	TYR	HD2	H	6.74	0.02	1
453	34	TYR	HE1	H	6.69	0.02	1
454	34	TYR	HE2	H	6.69	0.02	1
456	34	TYR	C	C	174.7	0.6	1
457	34	TYR	CA	C	57.6	0.6	1
458	34	TYR	CB	C	41.0	0.6	1
465	34	TYR	N	N	118.6	0.3	1
466	35	LYS	H	H	9.99	0.02	1
467	35	LYS	HA	H	4.84	0.02	1
468	35	LYS	HB2	H	1.82	0.02	1
469	35	LYS	HB3	H	1.59	0.02	1
470	35	LYS	HG2	H	1.18	0.02	1

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471	35	LYS	HG3	H	1.18	0.02	1
472	35	LYS	HD2	H	1.48	0.02	1
473	35	LYS	HD3	H	1.48	0.02	1
474	35	LYS	HE2	H	2.89	0.02	1
475	35	LYS	HE3	H	2.89	0.02	1
477	35	LYS	C	C	174.1	0.6	1
478	35	LYS	CA	C	54.2	0.6	1
479	35	LYS	CB	C	36.3	0.6	1
480	35	LYS	CG	C	24.1	0.6	1
481	35	LYS	CD	C	29.6	0.6	1
482	35	LYS	CE	C	41.6	0.6	1
483	35	LYS	N	N	119.7	0.3	1
485	36	GLN	H	H	8.77	0.02	1
486	36	GLN	HA	H	4.67	0.02	1
487	36	GLN	HB2	H	2.06	0.02	1
488	36	GLN	HB3	H	2.06	0.02	1
489	36	GLN	HG2	H	2.35	0.02	1
490	36	GLN	HG3	H	2.35	0.02	1
491	36	GLN	HE21	H	7.50	0.02	2
492	36	GLN	HE22	H	6.55	0.02	2
493	36	GLN	C	C	176.3	0.6	1
494	36	GLN	CA	C	56.7	0.6	1
495	36	GLN	CB	C	28.6	0.6	1
496	36	GLN	CG	C	33.7	0.6	1
498	36	GLN	N	N	124.9	0.3	1
499	36	GLN	NE2	N	110.9	0.3	1
500	37	GLU	H	H	8.96	0.02	1
501	37	GLU	HA	H	4.51	0.02	1
502	37	GLU	HB2	H	1.96	0.02	2
503	37	GLU	HB3	H	1.80	0.02	2
504	37	GLU	HG2	H	2.11	0.02	1
505	37	GLU	HG3	H	2.11	0.02	1
506	37	GLU	C	C	176.2	0.6	1
507	37	GLU	CA	C	56.0	0.6	1
508	37	GLU	CB	C	31.9	0.6	1
509	37	GLU	CG	C	36.6	0.6	1
511	37	GLU	N	N	130.2	0.3	1
512	38	GLY	H	H	9.20	0.02	1
513	38	GLY	HA2	H	3.69	0.02	1
514	38	GLY	HA3	H	4.05	0.02	1
515	38	GLY	C	C	174.7	0.6	1
516	38	GLY	CA	C	47.4	0.6	1
517	38	GLY	N	N	118.4	0.3	1
518	39	ASP	H	H	8.81	0.02	1
519	39	ASP	HA	H	4.69	0.02	1
520	39	ASP	HB2	H	2.78	0.02	1
521	39	ASP	HB3	H	2.78	0.02	1
522	39	ASP	C	C	175.2	0.6	1
523	39	ASP	CA	C	54.2	0.6	1
524	39	ASP	CB	C	41.0	0.6	1
526	39	ASP	N	N	127.1	0.3	1
527	40	LYS	H	H	7.86	0.02	1
528	40	LYS	HA	H	4.71	0.02	1
529	40	LYS	HB2	H	1.86	0.02	1
530	40	LYS	HB3	H	1.86	0.02	1
531	40	LYS	HG2	H	1.52	0.02	1
532	40	LYS	HG3	H	1.52	0.02	1
533	40	LYS	HD2	H	1.69	0.02	1

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534	40	LYS	HD3	H	1.69	0.02	1
535	40	LYS	HE2	H	3.02	0.02	1
536	40	LYS	HE3	H	3.02	0.02	1
538	40	LYS	C	C	175.4	0.6	1
539	40	LYS	CA	C	54.8	0.6	1
540	40	LYS	CB	C	36.3	0.6	1
541	40	LYS	CG	C	24.9	0.6	1
542	40	LYS	CD	C	29.0	0.6	1
543	40	LYS	CE	C	42.0	0.6	1
544	40	LYS	N	N	118.0	0.3	1
546	41	CYS	H	H	8.95	0.02	1
547	41	CYS	HA	H	5.29	0.02	1
548	41	CYS	HB2	H	3.00	0.02	1
549	41	CYS	HB3	H	2.58	0.02	1
551	41	CYS	C	C	174.4	0.6	1
552	41	CYS	CA	C	55.0	0.6	1
553	41	CYS	CB	C	41.9	0.6	1
554	41	CYS	N	N	119.8	0.3	1
555	42	VAL	H	H	9.35	0.02	1
556	42	VAL	HA	H	4.88	0.02	1
557	42	VAL	HB	H	2.32	0.02	1
558	42	VAL	HG1	H	1.00	0.02	1
559	42	VAL	HG2	H	0.88	0.02	1
560	42	VAL	C	C	175.6	0.6	1
561	42	VAL	CA	C	59.2	0.6	1
562	42	VAL	CB	C	35.2	0.6	1
563	42	VAL	CG1	C	21.6	0.6	1
564	42	VAL	CG2	C	19.3	0.6	1
565	42	VAL	N	N	118.7	0.3	1
566	43	GLU	H	H	9.18	0.02	1
567	43	GLU	HA	H	3.34	0.02	1
568	43	GLU	HB2	H	1.84	0.02	2
569	43	GLU	HB3	H	1.75	0.02	2
570	43	GLU	HG2	H	2.15	0.02	2
571	43	GLU	HG3	H	2.07	0.02	2
572	43	GLU	C	C	175.4	0.6	1
573	43	GLU	CA	C	59.0	0.6	1
574	43	GLU	CB	C	29.8	0.6	1
575	43	GLU	CG	C	36.8	0.6	1
577	43	GLU	N	N	123.4	0.3	1
578	44	ASN	H	H	8.25	0.02	1
579	44	ASN	HA	H	4.95	0.02	1
580	44	ASN	HB2	H	2.72	0.02	2
581	44	ASN	HB3	H	1.97	0.02	2
582	44	ASN	HD21	H	8.07	0.02	2
583	44	ASN	HD22	H	7.24	0.02	2
584	44	ASN	C	C	?	?	?
585	44	ASN	CA	C	48.5	0.6	1
586	44	ASN	CB	C	39.2	0.6	1
588	44	ASN	N	N	120.2	0.3	1
589	44	ASN	ND2	N	112.1	0.3	1
590	45	PRO	HA	H	4.40	0.02	1
591	45	PRO	HB2	H	2.31	0.02	1
592	45	PRO	HB3	H	1.95	0.02	1
593	45	PRO	HG2	H	1.97	0.02	1
594	45	PRO	HG3	H	1.97	0.02	1
595	45	PRO	HD2	H	3.86	0.02	2
596	45	PRO	HD3	H	3.81	0.02	2



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597	45	PRO	C	C	176.2	0.6	1
598	45	PRO	CA	C	63.7	0.6	1
599	45	PRO	CB	C	32.6	0.6	1
600	45	PRO	CG	C	26.8	0.6	1
601	45	PRO	CD	C	50.9	0.6	1
603	46	ASN	H	H	7.47	0.02	1
604	46	ASN	HA	H	5.09	0.02	1
605	46	ASN	HB2	H	2.72	0.02	2
606	46	ASN	HB3	H	2.43	0.02	2
607	46	ASN	HD21	H	7.57	0.02	1
608	46	ASN	HD22	H	6.91	0.02	1
609	46	ASN	C	C	?	?	?
610	46	ASN	CA	C	51.6	0.6	1
611	46	ASN	CB	C	40.1	0.6	1
613	46	ASN	N	N	114.0	0.3	1
614	46	ASN	ND2	N	112.6	0.3	1
615	47	PRO	HA	H	4.34	0.02	1
616	47	PRO	HB2	H	1.97	0.02	1
617	47	PRO	HB3	H	1.77	0.02	1
618	47	PRO	HG2	H	1.96	0.02	1
619	47	PRO	HG3	H	1.96	0.02	1
620	47	PRO	HD2	H	3.52	0.02	2
621	47	PRO	HD3	H	3.39	0.02	2
622	47	PRO	C	C	175.5	0.6	1
623	47	PRO	CA	C	63.7	0.6	1
624	47	PRO	CB	C	32.4	0.6	1
625	47	PRO	CG	C	27.5	0.6	1
626	47	PRO	CD	C	49.8	0.6	1
628	48	THR	H	H	8.28	0.02	1
629	48	THR	HA	H	4.68	0.02	1
630	48	THR	HB	H	4.32	0.02	1
632	48	THR	HG2	H	1.13	0.02	1
633	48	THR	C	C	174.5	0.6	1
634	48	THR	CA	C	59.1	0.6	1
635	48	THR	CB	C	69.9	0.6	1
636	48	THR	CG2	C	19.3	0.6	1
637	48	THR	N	N	112.4	0.3	1
638	49	CYS	H	H	9.35	0.02	1
639	49	CYS	HA	H	4.44	0.02	1
640	49	CYS	HB2	H	2.64	0.02	1
641	49	CYS	HB3	H	3.14	0.02	1
643	49	CYS	C	C	175.8	0.6	1
644	49	CYS	CA	C	55.5	0.6	1
645	49	CYS	CB	C	37.7	0.6	1
646	49	CYS	N	N	125.3	0.3	1
647	50	ASN	H	H	8.36	0.02	1
648	50	ASN	HA	H	4.60	0.02	1
649	50	ASN	HB2	H	2.88	0.02	1
650	50	ASN	HB3	H	2.70	0.02	1
651	50	ASN	HD21	H	7.60	0.02	2
652	50	ASN	HD22	H	6.97	0.02	2
653	50	ASN	C	C	174.4	0.6	1
654	50	ASN	CA	C	54.8	0.6	1
655	50	ASN	CB	C	38.9	0.6	1
657	50	ASN	N	N	116.4	0.3	1
658	50	ASN	ND2	N	113.6	0.3	1
659	51	GLU	H	H	7.43	0.02	1
660	51	GLU	HA	H	4.64	0.02	1

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661	51	GLU	HB2	H	1.96	0.02	2
662	51	GLU	HB3	H	1.81	0.02	2
663	51	GLU	HG2	H	2.12	0.02	1
664	51	GLU	HG3	H	2.12	0.02	1
665	51	GLU	C	C	176.1	0.6	1
666	51	GLU	CA	C	55.3	0.6	1
667	51	GLU	CB	C	31.5	0.6	1
668	51	GLU	CG	C	36.1	0.6	1
670	51	GLU	N	N	119.2	0.3	1
671	52	ASN	H	H	9.57	0.02	1
672	52	ASN	HA	H	4.45	0.02	1
673	52	ASN	HB2	H	3.14	0.02	1
674	52	ASN	HB3	H	2.64	0.02	1
675	52	ASN	HD21	H	6.99	0.02	1
676	52	ASN	HD22	H	7.71	0.02	1
677	52	ASN	C	C	176.4	0.6	1
678	52	ASN	CA	C	54.2	0.6	1
679	52	ASN	CB	C	37.8	0.6	1
681	52	ASN	N	N	125.4	0.3	1
682	52	ASN	ND2	N	112.8	0.3	1
683	53	ASN	H	H	9.31	0.02	1
684	53	ASN	HA	H	4.64	0.02	1
685	53	ASN	HB2	H	3.25	0.02	1
686	53	ASN	HB3	H	2.31	0.02	1
687	53	ASN	HD21	H	6.95	0.02	1
688	53	ASN	HD22	H	6.26	0.02	1
689	53	ASN	C	C	176.2	0.6	1
690	53	ASN	CA	C	54.1	0.6	1
691	53	ASN	CB	C	39.2	0.6	1
693	53	ASN	N	N	119.3	0.3	1
694	53	ASN	ND2	N	111.6	0.3	1
695	54	GLY	H	H	7.92	0.02	1
696	54	GLY	HA2	H	4.28	0.02	1
697	54	GLY	HA3	H	3.67	0.02	1
698	54	GLY	C	C	173.0	0.6	1
699	54	GLY	CA	C	46.3	0.6	1
700	54	GLY	N	N	106.3	0.3	1
701	55	GLY	H	H	8.06	0.02	1
702	55	GLY	HA2	H	4.39	0.02	1
703	55	GLY	HA3	H	3.47	0.02	1
704	55	GLY	C	C	175.1	0.6	1
705	55	GLY	CA	C	44.4	0.6	1
706	55	GLY	N	N	105.3	0.3	1
707	56	CYS	H	H	7.77	0.02	1
708	56	CYS	HA	H	4.39	0.02	1
709	56	CYS	HB2	H	3.12	0.02	1
710	56	CYS	HB3	H	2.81	0.02	1
712	56	CYS	C	C	175.2	0.6	1
713	56	CYS	CA	C	53.1	0.6	1
714	56	CYS	CB	C	36.6	0.6	1
715	56	CYS	N	N	117.6	0.3	1
716	57	ASP	H	H	8.37	0.02	1
717	57	ASP	HA	H	3.98	0.02	1
718	57	ASP	HB2	H	2.24	0.02	2
719	57	ASP	HB3	H	2.03	0.02	2
720	57	ASP	C	C	176.0	0.6	1
721	57	ASP	CA	C	56.0	0.6	1
722	57	ASP	CB	C	45.0	0.6	1

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724	57	ASP	N	N	122.3	0.3	1
725	58	ALA	H	H	8.48	0.02	1
726	58	ALA	HA	H	3.98	0.02	1
727	58	ALA	HB	H	1.41	0.02	1
728	58	ALA	C	C	178.9	0.6	1
729	58	ALA	CA	C	55.6	0.6	1
730	58	ALA	CB	C	19.1	0.6	1
731	58	ALA	N	N	127.2	0.3	1
732	59	ASP	H	H	9.11	0.02	1
733	59	ASP	HA	H	4.90	0.02	1
734	59	ASP	HB2	H	2.44	0.02	1
735	59	ASP	HB3	H	2.82	0.02	1
736	59	ASP	C	C	174.6	0.6	1
737	59	ASP	CA	C	53.9	0.6	1
738	59	ASP	CB	C	40.7	0.6	1
740	59	ASP	N	N	116.7	0.3	1
741	60	ALA	H	H	7.94	0.02	1
742	60	ALA	HA	H	5.13	0.02	1
743	60	ALA	HB	H	1.27	0.02	1
744	60	ALA	C	C	176.3	0.6	1
745	60	ALA	CA	C	50.5	0.6	1
746	60	ALA	CB	C	21.3	0.6	1
747	60	ALA	N	N	121.9	0.3	1
748	61	LYS	H	H	9.06	0.02	1
749	61	LYS	HA	H	4.54	0.02	1
750	61	LYS	HB2	H	1.77	0.02	1
751	61	LYS	HB3	H	1.77	0.02	1
752	61	LYS	HG2	H	1.31	0.02	2
753	61	LYS	HG3	H	1.39	0.02	2
754	61	LYS	HD2	H	1.72	0.02	2
755	61	LYS	HD3	H	1.63	0.02	2
756	61	LYS	HE2	H	2.92	0.02	2
757	61	LYS	HE3	H	2.92	0.02	2
759	61	LYS	C	C	175.7	0.6	1
760	61	LYS	CA	C	55.1	0.6	1
761	61	LYS	CB	C	34.1	0.6	1
762	61	LYS	CG	C	24.6	0.6	1
763	61	LYS	CD	C	29.1	0.6	1
764	61	LYS	CE	C	42.1	0.6	1
765	61	LYS	N	N	122.5	0.3	1
767	62	CYS	H	H	9.19	0.02	1
768	62	CYS	HA	H	5.32	0.02	1
769	62	CYS	HB2	H	2.44	0.02	1
770	62	CYS	HB3	H	2.80	0.02	1
772	62	CYS	C	C	174.5	0.6	1
773	62	CYS	CA	C	56.1	0.6	1
774	62	CYS	CB	C	37.6	0.6	1
775	62	CYS	N	N	131.5	0.3	1
776	63	THR	H	H	9.23	0.02	1
777	63	THR	HA	H	4.51	0.02	1
778	63	THR	HB	H	4.01	0.02	1
780	63	THR	HG2	H	1.16	0.02	1
781	63	THR	C	C	172.2	0.6	1
782	63	THR	CA	C	62.8	0.6	1
783	63	THR	CB	C	71.5	0.6	1
784	63	THR	CG2	C	22.0	0.6	1
785	63	THR	N	N	125.9	0.3	1
786	64	GLU	H	H	8.61	0.02	1

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787	64	GLU	HA	H	5.12	0.02	1
788	64	GLU	HB2	H	1.90	0.02	1
789	64	GLU	HB3	H	1.90	0.02	1
790	64	GLU	HG2	H	2.30	0.02	1
791	64	GLU	HG3	H	2.30	0.02	1
792	64	GLU	C	C	?	?	?
793	64	GLU	CA	C	54.4	0.6	1
794	64	GLU	CB	C	32.0	0.6	1
795	64	GLU	CG	C	36.8	0.6	1
797	64	GLU	N	N	123.0	0.3	1
798	65	GLU	H	H	8.76	0.02	1
799	65	GLU	HA	H	4.60	0.02	1
800	65	GLU	HB2	H	2.01	0.02	2
801	65	GLU	HB3	H	1.86	0.02	2
802	65	GLU	HG2	H	2.15	0.02	1
803	65	GLU	HG3	H	2.15	0.02	1
804	65	GLU	C	C	?	?	?
805	65	GLU	CA	C	55.1	0.6	1
806	65	GLU	CB	C	32.9	0.6	1
807	65	GLU	CG	C	36.1	0.6	1
809	65	GLU	N	N	123.0	0.3	1
810	66	ASP	H	H	8.79	0.02	1
811	66	ASP	HA	H	4.80	0.02	1
812	66	ASP	HB2	H	2.80	0.02	2
813	66	ASP	HB3	H	2.58	0.02	2
814	66	ASP	C	C	176.4	0.6	1
815	66	ASP	CA	C	54.8	0.6	1
816	66	ASP	CB	C	41.2	0.6	1
818	66	ASP	N	N	123.9	0.3	1
819	67	SER	H	H	8.38	0.02	1
820	67	SER	HA	H	4.55	0.02	1
821	67	SER	HB2	H	3.83	0.02	2
822	67	SER	HB3	H	3.70	0.02	2
824	67	SER	C	C	175.4	0.6	1
825	67	SER	CA	C	58.0	0.6	1
826	67	SER	CB	C	64.6	0.6	1
827	67	SER	N	N	119.1	0.3	1
828	68	GLY	H	H	8.65	0.02	1
829	68	GLY	HA2	H	4.13	0.02	2
830	68	GLY	HA3	H	3.85	0.02	2
831	68	GLY	C	C	175.0	0.6	1
832	68	GLY	CA	C	46.0	0.6	1
833	68	GLY	N	N	112.2	0.3	1
834	69	SER	H	H	8.58	0.02	1
835	69	SER	HA	H	4.42	0.02	1
836	69	SER	HB2	H	3.87	0.02	1
837	69	SER	HB3	H	3.87	0.02	1
839	69	SER	C	C	174.8	0.6	1
840	69	SER	CA	C	59.1	0.6	1
841	69	SER	CB	C	63.4	0.6	1
842	69	SER	N	N	117.7	0.3	1
843	70	ASN	H	H	8.39	0.02	1
844	70	ASN	HA	H	4.74	0.02	1
845	70	ASN	HB2	H	2.94	0.02	2
846	70	ASN	HB3	H	2.77	0.02	2
847	70	ASN	HD21	H	?	?	?
848	70	ASN	HD22	H	?	?	?
849	70	ASN	C	C	?	?	?

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850	70	ASN	CA	C	53.8	0.6	1
851	70	ASN	CB	C	38.9	0.6	1
853	70	ASN	N	N	118.4	0.3	1
854	70	ASN	ND2	N	?	?	?
855	71	GLY	H	H	7.88	0.02	1
856	71	GLY	HA2	H	4.07	0.02	1
857	71	GLY	HA3	H	4.07	0.02	1
858	71	GLY	C	C	173.7	0.6	1
859	71	GLY	CA	C	45.0	0.6	1
860	71	GLY	N	N	108.1	0.3	1
861	72	LYS	H	H	8.39	0.02	1
862	72	LYS	HA	H	4.84	0.02	1
863	72	LYS	HB2	H	1.75	0.02	2
864	72	LYS	HB3	H	1.59	0.02	2
865	72	LYS	HG2	H	1.46	0.02	2
866	72	LYS	HG3	H	1.38	0.02	2
867	72	LYS	HD2	H	1.66	0.02	1
868	72	LYS	HD3	H	1.66	0.02	1
869	72	LYS	HE2	H	2.93	0.02	1
870	72	LYS	HE3	H	2.93	0.02	1
872	72	LYS	C	C	173.7	0.6	1
873	72	LYS	CA	C	55.4	0.6	1
874	72	LYS	CB	C	35.0	0.6	1
875	72	LYS	CG	C	24.9	0.6	1
876	72	LYS	CD	C	28.9	0.6	1
877	72	LYS	CE	C	42.2	0.6	1
878	72	LYS	N	N	120.8	0.3	1
880	73	LYS	H	H	8.81	0.02	1
881	73	LYS	HA	H	4.64	0.02	1
882	73	LYS	HB2	H	1.71	0.02	1
883	73	LYS	HB3	H	1.71	0.02	1
884	73	LYS	HG2	H	1.36	0.02	2
885	73	LYS	HG3	H	1.25	0.02	2
886	73	LYS	HD2	H	1.63	0.02	1
887	73	LYS	HD3	H	1.63	0.02	1
888	73	LYS	HE2	H	2.89	0.02	1
889	73	LYS	HE3	H	2.89	0.02	1
891	73	LYS	C	C	175.3	0.6	1
892	73	LYS	CA	C	55.0	0.6	1
893	73	LYS	CB	C	35.4	0.6	1
894	73	LYS	CG	C	24.6	0.6	1
895	73	LYS	CD	C	?	?	?
896	73	LYS	CE	C	?	?	?
897	73	LYS	N	N	122.6	0.3	1
899	74	ILE	H	H	8.54	0.02	1
900	74	ILE	HA	H	4.83	0.02	1
901	74	ILE	HB	H	1.91	0.02	1
902	74	ILE	HG12	H	1.50	0.02	2
903	74	ILE	HG13	H	1.50	0.02	2
904	74	ILE	HG2	H	1.00	0.02	1
905	74	ILE	HD1	H	0.57	0.02	1
906	74	ILE	C	C	176.2	0.6	1
907	74	ILE	CA	C	58.4	0.6	1
908	74	ILE	CB	C	38.4	0.6	1
909	74	ILE	CG1	C	27.1	0.6	1
910	74	ILE	CG2	C	19.0	0.6	1
911	74	ILE	CD1	C	10.3	0.6	1
912	74	ILE	N	N	126.8	0.3	1

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913	75	THR	H	H	8.88	0.02	1
914	75	THR	HA	H	4.51	0.02	1
915	75	THR	HB	H	4.01	0.02	1
917	75	THR	HG2	H	1.15	0.02	1
918	75	THR	C	C	172.4	0.6	1
919	75	THR	CA	C	61.2	0.6	1
920	75	THR	CB	C	71.5	0.6	1
921	75	THR	CG2	C	22.0	0.6	1
922	75	THR	N	N	120.3	0.3	1
923	76	CYS	H	H	8.82	0.02	1
924	76	CYS	HA	H	5.53	0.02	1
925	76	CYS	HB2	H	3.28	0.02	1
926	76	CYS	HB3	H	2.69	0.02	1
928	76	CYS	C	C	174.7	0.6	1
929	76	CYS	CA	C	52.2	0.6	1
930	76	CYS	CB	C	40.6	0.6	1
931	76	CYS	N	N	121.5	0.3	1
932	77	GLU	H	H	8.64	0.02	1
933	77	GLU	HA	H	4.74	0.02	1
934	77	GLU	HB2	H	1.94	0.02	1
935	77	GLU	HB3	H	1.94	0.02	1
936	77	GLU	HG2	H	1.83	0.02	1
937	77	GLU	HG3	H	1.83	0.02	1
938	77	GLU	C	C	175.7	0.6	1
939	77	GLU	CA	C	54.7	0.6	1
940	77	GLU	CB	C	33.1	0.6	1
941	77	GLU	CG	C	36.1	0.6	1
943	77	GLU	N	N	123.0	0.3	1
944	78	CYS	H	H	9.71	0.02	1
945	78	CYS	HA	H	4.48	0.02	1
946	78	CYS	HB2	H	2.63	0.02	1
947	78	CYS	HB3	H	3.30	0.02	1
949	78	CYS	C	C	175.8	0.6	1
950	78	CYS	CA	C	57.4	0.6	1
951	78	CYS	CB	C	39.2	0.6	1
952	78	CYS	N	N	129.1	0.3	1
953	79	THR	H	H	8.20	0.02	1
954	79	THR	HA	H	4.25	0.02	1
955	79	THR	HB	H	4.25	0.02	1
957	79	THR	HG2	H	1.25	0.02	1
958	79	THR	C	C	176.2	0.6	1
959	79	THR	CA	C	63.4	0.6	1
960	79	THR	CB	C	70.1	0.6	1
961	79	THR	CG2	C	22.2	0.6	1
962	79	THR	N	N	114.7	0.3	1
963	80	LYS	H	H	8.56	0.02	1
964	80	LYS	HA	H	4.50	0.02	1
965	80	LYS	HB2	H	1.79	0.02	1
966	80	LYS	HB3	H	1.79	0.02	1
967	80	LYS	HG2	H	1.47	0.02	2
968	80	LYS	HG3	H	1.64	0.02	2
969	80	LYS	HD2	H	1.70	0.02	1
970	80	LYS	HD3	H	1.70	0.02	1
971	80	LYS	HE2	H	2.94	0.02	1
972	80	LYS	HE3	H	2.94	0.02	1
974	80	LYS	C	C	?	?	?
975	80	LYS	CA	C	55.4	0.6	1
976	80	LYS	CB	C	30.9	0.6	1

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977	80	LYS	CG	C	25.6	0.6	1
978	80	LYS	CD	C	29.1	0.6	1
979	80	LYS	CE	C	42.1	0.6	1
980	80	LYS	N	N	124.8	0.3	1
982	81	PRO	HA	H	4.26	0.02	1
983	81	PRO	HB2	H	2.27	0.02	1
984	81	PRO	HB3	H	1.87	0.02	1
985	81	PRO	HG2	H	2.10	0.02	2
986	81	PRO	HG3	H	2.04	0.02	2
987	81	PRO	HD2	H	3.90	0.02	2
988	81	PRO	HD3	H	3.63	0.02	2
989	81	PRO	C	C	176.9	0.6	1
990	81	PRO	CA	C	64.2	0.6	1
991	81	PRO	CB	C	31.9	0.6	1
992	81	PRO	CG	C	27.8	0.6	1
993	81	PRO	CD	C	50.8	0.6	1
995	82	ASP	H	H	8.78	0.02	1
996	82	ASP	HA	H	4.25	0.02	1
997	82	ASP	HB2	H	2.88	0.02	2
998	82	ASP	HB3	H	2.77	0.02	2
999	82	ASP	C	C	?	?	?
1000	82	ASP	CA	C	55.1	0.6	1
1001	82	ASP	CB	C	40.0	0.6	1
1003	82	ASP	N	N	117.9	0.3	1
1004	83	SER	H	H	7.39	0.02	1
1005	83	SER	HA	H	4.47	0.02	1
1006	83	SER	HB2	H	3.55	0.02	2
1007	83	SER	HB3	H	3.50	0.02	2
1009	83	SER	C	C	175.4	0.6	1
1010	83	SER	CA	C	57.4	0.6	1
1011	83	SER	CB	C	65.9	0.6	1
1012	83	SER	N	N	112.9	0.3	1
1013	84	TYR	H	H	8.72	0.02	1
1014	84	TYR	HA	H	5.01	0.02	1
1015	84	TYR	HB2	H	2.93	0.02	2
1016	84	TYR	HB3	H	2.75	0.02	2
1017	84	TYR	HD1	H	6.92	0.02	1
1018	84	TYR	HD2	H	6.92	0.02	1
1019	84	TYR	HE1	H	6.69	0.02	1
1020	84	TYR	HE2	H	6.69	0.02	1
1022	84	TYR	C	C	?	?	?
1023	84	TYR	CA	C	54.6	0.6	1
1024	84	TYR	CB	C	39.7	0.6	1
1031	84	TYR	N	N	122.2	0.3	1
1032	85	PRO	HA	H	5.09	0.02	1
1033	85	PRO	HB2	H	2.25	0.02	2
1034	85	PRO	HB3	H	1.72	0.02	2
1035	85	PRO	HG2	H	2.22	0.02	1
1036	85	PRO	HG3	H	2.22	0.02	1
1037	85	PRO	HD2	H	3.85	0.02	1
1038	85	PRO	HD3	H	3.85	0.02	1
1039	85	PRO	C	C	176.9	0.6	1
1040	85	PRO	CA	C	62.9	0.6	1
1041	85	PRO	CB	C	32.8	0.6	1
1042	85	PRO	CG	C	27.3	0.6	1
1043	85	PRO	CD	C	50.6	0.6	1
1045	86	LEU	H	H	8.51	0.02	1
1046	86	LEU	HA	H	4.77	0.02	1

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1047	86	LEU	HB2	H	1.76	0.02	1
1048	86	LEU	HB3	H	1.76	0.02	1
1049	86	LEU	HG	H	1.85	0.02	1
1050	86	LEU	HD1	H	1.00	0.02	1
1051	86	LEU	HD2	H	1.00	0.02	1
1052	86	LEU	C	C	177.9	0.6	1
1053	86	LEU	CA	C	54.5	0.6	1
1054	86	LEU	CB	C	44.8	0.6	1
1055	86	LEU	CG	C	29.0	0.6	1
1056	86	LEU	CD1	C	25.7	0.6	1
1057	86	LEU	CD2	C	25.7	0.6	1
1058	86	LEU	N	N	120.2	0.3	1
1059	87	PHE	H	H	9.29	0.02	1
1060	87	PHE	HA	H	4.16	0.02	1
1061	87	PHE	HB2	H	3.26	0.02	2
1062	87	PHE	HB3	H	3.19	0.02	2
1063	87	PHE	HD1	H	7.31	0.02	1
1064	87	PHE	HD2	H	7.31	0.02	1
1065	87	PHE	HE1	H	7.70	0.02	1
1066	87	PHE	HE2	H	7.70	0.02	1
1067	87	PHE	HZ	H	7.67	0.02	1
1068	87	PHE	C	C	176.4	0.6	1
1069	87	PHE	CA	C	59.3	0.6	1
1070	87	PHE	CB	C	36.9	0.6	1
1077	87	PHE	N	N	122.6	0.3	1
1078	88	ASP	H	H	8.93	0.02	1
1079	88	ASP	HA	H	4.33	0.02	1
1080	88	ASP	HB2	H	3.11	0.02	2
1081	88	ASP	HB3	H	3.02	0.02	2
1082	88	ASP	C	C	175.2	0.6	1
1083	88	ASP	CA	C	56.3	0.6	1
1084	88	ASP	CB	C	39.9	0.6	1
1086	88	ASP	N	N	111.3	0.3	1
1087	89	GLY	H	H	7.87	0.02	1
1088	89	GLY	HA2	H	3.48	0.02	1
1089	89	GLY	HA3	H	4.08	0.02	1
1090	89	GLY	C	C	174.7	0.6	1
1091	89	GLY	CA	C	46.0	0.6	1
1092	89	GLY	N	N	102.0	0.3	1
1093	90	ILE	H	H	7.17	0.02	1
1094	90	ILE	HA	H	4.39	0.02	1
1095	90	ILE	HB	H	1.52	0.02	1
1096	90	ILE	HG12	H	0.65	0.02	2
1097	90	ILE	HG13	H	0.65	0.02	2
1098	90	ILE	HG2	H	1.05	0.02	1
1099	90	ILE	HD1	H	0.51	0.02	1
1100	90	ILE	C	C	175.1	0.6	1
1101	90	ILE	CA	C	64.2	0.6	1
1102	90	ILE	CB	C	35.9	0.6	1
1103	90	ILE	CG1	C	25.5	0.6	1
1104	90	ILE	CG2	C	16.9	0.6	1
1105	90	ILE	CD1	C	14.9	0.6	1
1106	90	ILE	N	N	113.3	0.3	1
1107	91	PHE	H	H	7.43	0.02	1
1108	91	PHE	HA	H	5.15	0.02	1
1109	91	PHE	HB2	H	2.40	0.02	1
1110	91	PHE	HB3	H	2.40	0.02	1
1111	91	PHE	HD1	H	7.00	0.02	1



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1112	91	PHE	HD2	H	7.00	0.02	1
1113	91	PHE	HE1	H	6.95	0.02	1
1114	91	PHE	HE2	H	6.95	0.02	1
1115	91	PHE	HZ	H	7.06	0.02	1
1116	91	PHE	C	C	175.1	0.6	1
1117	91	PHE	CA	C	56.5	0.6	1
1118	91	PHE	CB	C	44.3	0.6	1
1125	91	PHE	N	N	114.0	0.3	1
1126	92	CYS	H	H	7.86	0.02	1
1127	92	CYS	HA	H	5.18	0.02	1
1128	92	CYS	HB2	H	2.57	0.02	1
1129	92	CYS	HB3	H	3.08	0.02	1
1131	92	CYS	C	C	174.1	0.6	1
1132	92	CYS	CA	C	54.1	0.6	1
1133	92	CYS	CB	C	44.7	0.6	1
1134	92	CYS	N	N	119.0	0.3	1
1135	93	SER	H	H	9.26	0.02	1
1136	93	SER	HA	H	4.23	0.02	1
1137	93	SER	HB2	H	3.90	0.02	1
1138	93	SER	HB3	H	3.90	0.02	1
1140	93	SER	C	C	?	?	?
1141	93	SER	CA	C	60.4	0.6	1
1142	93	SER	CB	C	63.7	0.6	1
1143	93	SER	N	N	118.3	0.3	1
1144	94	SER	H	H	8.04	0.02	1
1145	94	SER	HA	H	4.59	0.02	1
1146	94	SER	HB2	H	3.91	0.02	1
1147	94	SER	HB3	H	3.91	0.02	1
1149	94	SER	C	C	?	?	?
1150	94	SER	CA	C	58.7	0.6	1
1151	94	SER	CB	C	64.0	0.6	1
1152	94	SER	N	N	114.2	0.3	1
1153	95	SER	H	H	?	?	?
1154	95	SER	HA	H	4.71	0.02	1
1155	95	SER	HB2	H	4.02	0.02	2
1156	95	SER	HB3	H	3.94	0.02	2
1158	95	SER	C	C	?	?	?
1159	95	SER	CA	C	58.4	0.6	1
1160	95	SER	CB	C	64.5	0.6	1
1161	95	SER	N	N	?	?	?
1162	96	ASN	H	H	?	?	?
1163	96	ASN	HA	H	4.61	0.02	1
1164	96	ASN	HB2	H	2.75	0.02	2
1165	96	ASN	HB3	H	2.60	0.02	2
1166	96	ASN	HD21	H	?	?	?
1167	96	ASN	HD22	H	?	?	?
1168	96	ASN	C	C	?	?	?
1169	96	ASN	CA	C	54.3	0.6	1
1170	96	ASN	CB	C	41.8	0.6	1
1172	96	ASN	N	N	?	?	?
1173	96	ASN	ND2	N	?	?	?

stop\_

# The following loop is used to define sets of Atom-shift assignment IDs that represent related ambiguous assignments taken from the above list of assigned chemical shifts. Each element in the set should be separated by a comma, as shown in the example below, and is the assignment ID for a chemical shift

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assignment that has been given as ambiguity code of 4 or 5. Each set indicates that the observed chemical shifts are related to the defined atoms, but have not been assigned uniquely to a specific atom in the set.

```
loop_  
  _Atom_shift_assign_ID_ambiguity  
  
#  
#   Sets of Atom-shift Assignment Ambiguities  
#  
#   -----  
# Example:  5,4,7  
#  
#           @  
stop_
```

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```
#####
#-----REMARKS-----#
#####
#
# PROTECTED BACKBONE AMIDE GROUPS
# (SLOWLY EXCHANGING IN D2O) FOR RESIDUES:
# GLY 17 , PHE 19 , GLU 27 , LYS 29 , LEU 31 ,
# TYR 34 , LYS 35 , VAL 42 , CYS 56 , ASP 57 ,
# ALA 60 , LYS 61 , THR 63 , THR 75 , GLU 77 ,
# LEU 86 , GLY 89 , ILE 90 , PHE 91
#
# BROAD HN SIGNALS IN [15-N]-HSQC OBSERVED FOR RESIDUES:
# VAL 8 , LYS 9 , LYS 10 , CYS 18 , ARG 20
#
# TWO BACKBONE HN CROSSPEAKS OBSERVED FOR RESIDUES:
# HIS 5 : 7.78,113.8 / 7.74,113.5
# GLN 6 : 7.44,122.6 / 7.40,122.4
#
# TWO AVERAGED NH*/HH* SIGNALS OBSERVED FOR RESIDUES:
# ARG 20 , ARG 25 : NOT SPECIFICALLY ASSIGNED
# TO INDIVIDUAL ARGININES
#
# LYS 29 NZ/HZ* SIGNAL: TENTATIVELY ASSIGNED TO
# LYS 29 (BURIED LYSINE SIDE CHAIN)
# BASED ON GREATER PROTECTION FROM H2O EXCHANGE
# THAN OTHER LYSINE NZ/HZ* SIGNALS
#
# ASPARAGINE SIDE CHAIN AMIDE SIGNALS:
# PROBABLE OVERLAPPING CROSSPEAKS ~112 PPM [15N]
# FOR ASN 1 , ASN 70 , ASN 96
#
#####
#
#####
```

Table A2. Supplementary: NMR experimental details

Experiment	Dimension	Nucleus	Complex Points [after LP]	Spectral width (Hz)	Acquisition Time (ms)	Carrier Frequency (ppm)	Instrument <sup>1</sup> H- frequency (MHz)	Solvent	Temperature (°C)	Temp- erature	Final data size (points)	Digital Resolution (Hz/ point)	Mixing time (ms)	Total time (hr)
2D NOESY	t1	<sup>1</sup> H	400	8000	50	4.74	600	D <sub>2</sub> O	25		1024	7.8	75-150	22
	t2	<sup>1</sup> H	2048	8000	256	4.74					2048	3.9		
2D NOESY	t1	<sup>1</sup> H	360	8000	45	4.74	600	H <sub>2</sub> O	25		1024	7.8	75	23
	t2	<sup>1</sup> H	2048	8000	256	4.74					2048	3.9		
2D ROESY	t1	<sup>1</sup> H	260	6000	43	4.74	500	D <sub>2</sub> O	25		1024	5.9	60	16
	t2	<sup>1</sup> H	2048	6000	341	4.74					2048	2.9		
2D ROESY	t1	<sup>1</sup> H	360	7000	54	4.74	500	H <sub>2</sub> O	25		1024	6.8	60	62
	t2	<sup>1</sup> H	2048	7000	293	4.74					2048	3.4		
3D [ <sup>15</sup> N]-NOESY-HSQ	t1	<sup>15</sup> N	36 [64]	2500	14.4	121.5	500	H <sub>2</sub> O	25		128	19.5	125	64
	t2	<sup>1</sup> H	180	7000	26	4.74					512	13.7		
	t3	<sup>1</sup> H	512	7000	73	4.74					512	13.7		
3D [ <sup>15</sup> N]-ROESY-HSQC	t1	<sup>15</sup> N	32	2500	12.8	121.5	500	H <sub>2</sub> O	25		128	19.5	60	87
	t2	<sup>1</sup> H	180	7000	26	4.74					512	13.7		
	t3	<sup>1</sup> H	512	7000	73	4.74					512	13.7		
3D [ <sup>13</sup> C]-HMQC-NOESY	t1	<sup>1</sup> H	160	7200	22	4.74	600	D <sub>2</sub> O	25		512	14.1	125	89
	t2	<sup>13</sup> C	96	10000	9.6	41.0					256	39.1		
	t3	<sup>1</sup> H	384	7200	53	4.74					1024	7.0		
4D [ <sup>13</sup> C]-HMQC-NOESY-[ <sup>13</sup> C]-HSQC	t1	<sup>13</sup> C	18 [24]	3360	5.4	40.2	600	D <sub>2</sub> O	25		64	52.5	125	105
	t2	<sup>1</sup> H	74	3600	21	3.00					256	14.1		
	t3	<sup>13</sup> C	18 [24]	3360	5.4	40.2					64	52.5		
	t4	<sup>1</sup> H	256	4500	57	3.00					256	17.6		

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Experiment (Reference)	Dimension	Nucleus	Complex Points [after LP]	Spectral width (Hz)	Acquisition Time (ms)	Carrier Frequency (ppm)	Instrument H-frequency (MHz)	Solvent	Temperature (°C)	Final data size (points)	Digital Res. (Hz/point)(ms)	Mixing time (hr)	Total time (hr)
4D [ <sup>13</sup> C]-HMQC-NOESY-[( <sup>15</sup> N)-HSQC													
	t1	<sup>13</sup> C	14 [24]	3360	4.2	40.2	600	H <sub>2</sub> O	25	64	52.5	125	96
	t2	<sup>1</sup> H	56	3600	15.6	3.00				128	28.1		
	t3	<sup>15</sup> N	14 [24]	1800	7.8	118.9				64	28.1		
	t4	<sup>1</sup> H	256	7400	34.6	4.74				256	29.0		
2D DQF-COSY													
	t1	<sup>1</sup> H	1000	6000	167	4.74	500	D <sub>2</sub> O	25	4096	1.5		31
	t2	<sup>1</sup> H	2048	6000	341	4.74				8192	0.7		
3D HNHA													
	t1	<sup>15</sup> N	35	2500	14	121.5	500	H <sub>2</sub> O	25	128	19.5		41
	t2	<sup>1</sup> H	80	3500	23	4.74				256	13.7		
	t3	<sup>1</sup> H	512	7000	73	4.74				512	13.7		
3D HNHB													
	t1	<sup>15</sup> N	24 [48]	2500	9.6	119.1	600	H <sub>2</sub> O	25	128	19.5		60
	t2	<sup>1</sup> H	90	8000	11.3	4.74				256	31.3		-71-
	t3	<sup>1</sup> H	512	8000	64	4.74				1024	7.8		
3D HN(CO)HB													
	t1	<sup>15</sup> N	28 [48]	1800	15.6	118.9	600	H <sub>2</sub> O	25	128	14.1		108
	t2	<sup>1</sup> H	128	8000	16	4.74				512	15.6		
	t3	<sup>1</sup> H	512	8000	64	4.74				512	15.6		
2D [ <sup>15</sup> N]-[ <sup>13</sup> Cy] Spin-echo HSQC													
	t1	<sup>15</sup> N	80	1800	44.4	118.9	600	H <sub>2</sub> O	25	256	7.0		13
	t2	<sup>1</sup> H	1312	8000	149	4.74				2048	3.9		
2D [ <sup>13</sup> C]-[ <sup>13</sup> Cy] Spin-echo HSQC													
	t1	<sup>15</sup> N	78	1800	43.3	118.9	600	H <sub>2</sub> O	25	256	7.0		26
	t2	<sup>1</sup> H	1216	8000	152	4.74				2048	3.9		
3D LRCH													
	t1	<sup>13</sup> C	34	3017	11.3	17.9	600	D <sub>2</sub> O	25	256	11.8		84
	t2	<sup>1</sup> H	57	4800	11.9	2.25				256	18.8		
	t3	<sup>1</sup> H	384	4000	96	2.25				1024	3.9		

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Experiment {Reference}	Dimension	Nucleus	Complex Points	Spectral width [after LP] (Hz)	Acquisition Time (ms)	Carrier Frequency (ppm)	Instrument 'H-frequency (MHz)	Solvent	Temper- ature (°C)	Final data size (points)	Digital Resolution (Hz/ point)	Mixing time (ms)	Total time (hr)
2D TOCSY	11	<sup>1</sup> H	360	8000	45	4.74	600	H <sub>2</sub> O	25	1024	7.8	66	18
	12	<sup>1</sup> H	2048	8000	256	4.74				2048	3.9		
2D [ <sup>15</sup> N]-HSQC	11	<sup>15</sup> N	360	4400	82	119.6	600	H <sub>2</sub> O	25	2048	2.1		14
	12	<sup>1</sup> H	1216	8000	152	4.74				4096	2.0		
2D [ <sup>13</sup> C]-HSQC	11	<sup>13</sup> C	400	12000	33.3	41.3	600	H <sub>2</sub> O	25	1024	11.7		2.7
	12	<sup>1</sup> H	1216	8000	152	4.74				4096	2.0		
3D [ <sup>15</sup> N]-TOCSY-HSQ	11	<sup>15</sup> N	38 [64]	2500	15.2	119.0	600	H <sub>2</sub> O	25	128	19.5	56	43
	12	<sup>1</sup> H	180	8000	22.5	4.74				512	15.6		
	13	<sup>1</sup> H	512	8000	64	4.74				512	15.6		
3D HICCH-TOCSY	11	<sup>1</sup> H	134	5500	24.4	4.74	500	D <sub>2</sub> O	25	512	10.7	17	65
	12	<sup>13</sup> C	128	8049	15.9	41.9				512	15.7		
	13	<sup>1</sup> H	416	5500	76	4.74				512	10.7		
3D CBCA(CO)NH	11	<sup>13</sup> C	38[64]	10000	3.8	41.3	600	H <sub>2</sub> O	25	128	78.1		21
	12	<sup>15</sup> N	26[36]	1800	14.4	118.9				128	14.1		
	13	<sup>1</sup> H	512	8000	64	4.74				512	15.6		
3D CBCANH	11	<sup>13</sup> C	63[128]	10000	6.3	41.3	600	H <sub>2</sub> O	25	512	19.5		5
	12	<sup>15</sup> N	26[52]	1800	14.4	118.9				128	14.1		
	13	<sup>1</sup> H	512	8000	64	4.74				256	31.3		
3D HNC0	11	<sup>15</sup> N	32[48]	1800	17.8	118.9	600	H <sub>2</sub> O	25	128	14.1		43
	12	<sup>13</sup> C	64	1811	35.3	176.0				256	7.1		
	13	<sup>1</sup> H	512	8000	64	4.74				512	15.6		

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TABLE B

```

! merozoite surface protein-1 (MSP-1) P. falciparum C-terminal fragment
! X-PLOR format
! 09-11-98
! --- noes + roes --approximate-- 570 total
!   long-range      135
!   medium-range     30
!   sequential      222
!   intraresidue     73
!   hydrogen_bonds  10  (20 restraints)
!
! pseudoatom corrections not used -- for R-6 averaging/summation
! AVERAGING:
! class rt6s SUM
! class nsam SUM
! class sing
! class hbnd
! types:
!   arom_          aromatic pair
!   meth_          methyl
!   dgm_           degenerate methylene
!   nsam_          non-stereospecifically-assigned methylene
!   sing_          single proton
!   _l             long-range          (j-i > 4)
!   _m             medium-range        (j-i =2-4)
!   _s             sequential          (j-i = 1)
!   _i             intraresidue
!   hbnd_          hydrogen_bonds
!
!   <residue-atom 1>      <residue-atom 2>      <dist-minus-plus>
! <type>
!
! class rt6s
!
! assign (resid 5 and name hb#) (resid 19 and name hd#) 3.6 3.6 0.0 !arom_l
! assign (resid 5 and name hb#) (resid 19 and name he#) 3.6 3.6 0.0 !arom_l
! assign (resid 15 and name ha) (resid 34 and name hd#) 3.6 3.6 0.0 !arom_l
! assign (resid 15 and name ha) (resid 34 and name he#) 3.6 3.6 0.0 !arom_l
! assign (resid 17 and name ha#) (resid 87 and name hd#) 5.5 5.5 0.0 !arom_l
! assign (resid 17 and name ha#) (resid 87 and name he#) 5.5 5.5 0.0 !arom_l
! assign (resid 19 and name hd#) (resid 20 and name hn) 5.5 5.5 0.0 !arom_s
! assign (resid 19 and name hd#) (resid 21 and name hn) 5.5 5.5 0.0 !arom_s
! assign (resid 19 and name hd#) (resid 21 and name hd2) 3.6 3.6 0.0 !arom_m
! assign (resid 19 and name hd#) (resid 22 and name hd#) 3.6 3.6 0.0 !arom_m
! assign (resid 19 and name hd#) (resid 86 and name hg) 5.5 5.5 0.0 !arom_l
! assign (resid 19 and name hd#) (resid 91 and name hb#) 3.6 3.6 0.0 !arom_l
! assign (resid 19 and name hd#) (resid 91 and name hd#) 3.6 3.6 0.0 !arom_l
! assign (resid 19 and name hd#) (resid 91 and name he#) 5.5 5.5 0.0 !arom_l
! assign (resid 19 and name he#) (resid 21 and name hd2) 5.5 5.5 0.0 !arom_m
! assign (resid 19 and name he#) (resid 22 and name hd#) 3.6 3.6 0.0 !arom_m
! assign (resid 19 and name he#) (resid 86 and name hg) 5.5 5.5 0.0 !arom_l
! assign (resid 19 and name he#) (resid 91 and name hb#) 3.6 3.6 0.0 !arom_l
! assign (resid 19 and name he#) (resid 91 and name hd#) 5.5 5.5 0.0 !arom_l
! assign (resid 31 and name hn) (resid 34 and name hd#) 3.6 3.6 0.0 !arom_m
! assign (resid 31 and name hg) (resid 34 and name hd#) 5.5 5.5 0.0 !arom_m
! assign (resid 31 and name hd#) (resid 34 and name hd#) 5.5 5.5 0.0 !arom_m
! assign (resid 31 and name hd1#) (resid 34 and name he#) 5.5 5.5 0.0 !arom_m
! assign (resid 31 and name hd2#) (resid 87 and name hd#) 2.8 2.8 0.0 !arom_l
! assign (resid 32 and name hn) (resid 34 and name hd#) 5.5 5.5 0.0 !arom_m
! assign (resid 34 and name hn) (resid 34 and name hd#) 3.6 3.6 0.0 !arom_l
! assign (resid 34 and name ha) (resid 34 and name hd#) 3.6 3.6 0.0 !arom_l
! assign (resid 34 and name hd#) (resid 35 and name hn) 3.6 3.6 0.0 !arom_s

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assign (resid 34 and name hd#)	(resid 42 and name hn)	5.5 5.5 0.0	!arom_l
assign (resid 34 and name hd#)	(resid 42 and name ha)	3.6 3.6 0.0	!arom_l
assign (resid 34 and name he#)	(resid 43 and name hn)	5.5 5.5 0.0	!arom_l
assign (resid 34 and name hd#)	(resid 43 and name hb#)	5.5 5.5 0.0	!arom_l
assign (resid 34 and name he#)	(resid 42 and name hn)	5.5 5.5 0.0	!arom_l
assign (resid 34 and name he#)	(resid 42 and name ha)	5.5 5.5 0.0	!arom_l
assign (resid 34 and name he#)	(resid 43 and name hb#)	5.5 5.5 0.0	!arom_l
assign (resid 34 and name he#)	(resid 43 and name hg#)	3.6 3.6 0.0	!arom_l
assign (resid 33 and name hn)	(resid 34 and name hd#)	3.6 3.6 0.0	!arom_s
assign (resid 36 and name hn)	(resid 31 and name hd#)	5.5 5.5 0.0	!arom_l
assign (resid 36 and name hb#)	(resid 37 and name hd#)	2.8 2.8 0.0	!arom_s
assign (resid 36 and name hb#)	(resid 37 and name he#)	3.6 3.6 0.0	!arom_s
assign (resid 36 and name hd#)	(resid 37 and name hd#)	3.6 3.6 0.0	!arom_s
assign (resid 36 and name hd#)	(resid 37 and name he#)	3.6 3.6 0.0	!arom_s
assign (resid 37 and name ha)	(resid 27 and name hd#)	2.8 2.8 0.0	!arom_i
assign (resid 37 and name hd#)	(resid 90 and name hb)	5.5 5.5 0.0	!arom_m
assign (resid 37 and name hd#)	(resid 90 and name hg2#)	5.5 5.5 0.0	!arom_m
assign (resid 37 and name hd#)	(resid 90 and name hd#)	3.6 3.6 0.0	!arom_m
assign (resid 37 and name hd#)	(resid 91 and name hb#)	3.6 3.6 0.0	!arom_m
assign (resid 37 and name hd#)	(resid 91 and name hd#)	2.8 2.8 0.0	!arom_m
assign (resid 37 and name hd#)	(resid 91 and name he#)	3.6 3.6 0.0	!arom_m
assign (resid 37 and name he#)	(resid 91 and name hd#)	5.5 5.5 0.0	!arom_m
assign (resid 37 and name he#)	(resid 91 and name he#)	5.5 5.5 0.0	!arom_m
assign (resid 91 and name hn)	(resid 91 and name hd#)	3.6 3.6 0.0	!arom_i
assign (resid 91 and name hd#)	(resid 92 and name hn)	5.5 5.5 0.0	!arom_s
assign (resid 2 and name hd#)	(resid 86 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 5 and name hb#)	(resid 86 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 17 and name hn)	(resid 90 and name hd#)	4.1 4.1 0.0	!meth_l
assign (resid 17 and name ha#)	(resid 90 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 29 and name hb#)	(resid 90 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 29 and name hg#)	(resid 90 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 29 and name hd#)	(resid 90 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 29 and name he#)	(resid 90 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 31 and name hn)	(resid 90 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 31 and name ha)	(resid 90 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 31 and name ha)	(resid 90 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name ha)	(resid 90 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name hb#)	(resid 90 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name hg)	(resid 90 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 40 and name hn)	(resid 40 and name he#)	5.5 5.5 0.0	!meth_i
assign (resid 47 and name ha)	(resid 48 and name hg2#)	5.5 5.5 0.0	!meth_s
assign (resid 47 and name hb1)	(resid 74 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 47 and name hg#)	(resid 74 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 48 and name hn)	(resid 48 and name hg2#)	3.6 3.6 0.0	!meth_i
assign (resid 48 and name hn)	(resid 74 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 48 and name ha)	(resid 74 and name hd#)	3.6 3.6 0.0	!meth_l
assign (resid 48 and name hg2#)	(resid 49 and name hn)	5.5 5.5 0.0	!meth_s
assign (resid 48 and name hg2#)	(resid 50 and name hn)	3.6 3.6 0.0	!meth_m
assign (resid 48 and name hg2#)	(resid 50 and name hb1)	5.5 5.5 0.0	!meth_m
assign (resid 48 and name hg2#)	(resid 51 and name hn)	3.1 3.1 0.0	!meth_m
assign (resid 48 and name hg2#)	(resid 51 and name ha)	3.6 3.6 0.0	!meth_m
assign (resid 48 and name hg2#)	(resid 51 and name hb#)	3.6 3.6 0.0	!meth_m
assign (resid 48 and name hg2#)	(resid 51 and name hg#)	5.5 5.5 0.0	!meth_m
assign (resid 48 and name hg2#)	(resid 74 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 49 and name hn)	(resid 74 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 49 and name ha)	(resid 74 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 54 and name hn)	(resid 74 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 54 and name ha#)	(resid 74 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 54 and name ha#)	(resid 74 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 56 and name hn)	(resid 60 and name hb#)	5.5 5.5 0.0	!meth_m
assign (resid 56 and name hn)	(resid 74 and name hg2#)	5.5 5.5 0.0	!meth_i
assign (resid 56 and name ha)	(resid 60 and name hb#)	5.5 5.5 0.0	!meth_m
assign (resid 56 and name ha)	(resid 90 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 56 and name hb1)	(resid 60 and name hb#)	3.6 3.6 0.0	!meth_m
assign (resid 57 and name hn)	(resid 60 and name hb#)	3.6 3.6 0.0	!meth_m



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assign (resid 57 and name ha)	(resid 60 and name hb#)	5.5	5.5	0.0	!meth_m
assign (resid 57 and name hb#)	(resid 60 and name hb#)	5.5	5.5	0.0	!meth_m
assign (resid 59 and name hb#)	(resid 59 and name hn)	3.6	3.6	0.0	!meth_s
assign (resid 59 and name hb#)	(resid 60 and name hn)	5.5	5.5	0.0	!meth_m
assign (resid 59 and name hn)	(resid 60 and name hb#)	5.5	5.5	0.0	!meth_s
assign (resid 60 and name hb#)	(resid 61 and name hn)	3.6	3.6	0.0	!meth_s
assign (resid 60 and name hb#)	(resid 76 and name ha)	5.5	5.5	0.0	!meth_l
assign (resid 60 and name hb#)	(resid 76 and name hb1)	5.5	5.5	0.0	!meth_l
assign (resid 60 and name hb#)	(resid 77 and name hn)	3.6	3.6	0.0	!meth_l
assign (resid 60 and name hb#)	(resid 77 and name ha)	5.5	5.5	0.0	!meth_l
assign (resid 60 and name hb#)	(resid 78 and name hn)	5.5	5.5	0.0	!meth_l
assign (resid 60 and name hb#)	(resid 78 and name hb1)	5.5	5.5	0.0	!meth_l
assign (resid 62 and name ha)	(resid 74 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 63 and name hn)	(resid 74 and name hg2#)	4.1	4.1	0.0	!meth_l
assign (resid 63 and name hn)	(resid 74 and name hd#)	3.6	3.6	0.0	!meth_l
assign (resid 63 and name hg2#)	(resid 64 and name hn)	3.6	3.6	0.0	!meth_s
assign (resid 64 and name ha)	(resid 74 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 64 and name ha)	(resid 74 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 64 and name hb#)	(resid 74 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 64 and name hg#)	(resid 74 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 72 and name hn)	(resid 72 and name he#)	3.6	3.6	0.0	!meth_i
assign (resid 72 and name hb#)	(resid 74 and name hd#)	5.5	5.5	0.0	!meth_m
assign (resid 73 and name hn)	(resid 74 and name hd#)	5.5	5.5	0.0	!meth_s
assign (resid 74 and name hn)	(resid 74 and name hg2#)	3.6	3.6	0.0	!meth_i
assign (resid 74 and name ha)	(resid 74 and name hg2#)	3.6	3.6	0.0	!meth_i
assign (resid 74 and name ha)	(resid 74 and name hd#)	3.6	3.6	0.0	!meth_i
assign (resid 74 and name hg2#)	(resid 75 and name ha)	5.5	5.5	0.0	!meth_s
assign (resid 75 and name hg2#)	(resid 76 and name hn)	3.6	3.6	0.0	!meth_s
assign (resid 79 and name hn)	(resid 79 and name hg2#)	3.6	3.6	0.0	!meth_i
assign (resid 79 and name hg2#)	(resid 80 and name hn)	3.6	3.6	0.0	!meth_s
assign (resid 95 and name ha)	(resid 86 and name hd#)	5.5	5.5	0.0	!meth_s
assign (resid 86 and name hd#)	(resid 87 and name hn)	5.5	5.5	0.0	!meth_s
assign (resid 86 and name hd#)	(resid 91 and name hb#)	5.5	5.5	0.0	!meth_l
assign (resid 86 and name hd#)	(resid 92 and name ha)	5.5	5.5	0.0	!meth_l
assign (resid 87 and name ha)	(resid 90 and name hd#)	5.5	5.5	0.0	!meth_m
assign (resid 87 and name hb#)	(resid 90 and name hg2#)	5.5	5.5	0.0	!meth_m
assign (resid 87 and name hb#)	(resid 90 and name hd#)	5.5	5.5	0.0	!meth_m
assign (resid 89 and name hn)	(resid 90 and name hg2#)	5.5	5.5	0.0	!meth_s
assign (resid 90 and name hn)	(resid 90 and name hg2#)	3.6	3.6	0.0	!meth_i
assign (resid 90 and name ha)	(resid 90 and name hg2#)	3.6	3.6	0.0	!meth_i
assign (resid 90 and name hg2#)	(resid 91 and name hn)	5.5	5.5	0.0	!meth_s
assign (resid 90 and name hd#)	(resid 91 and name hn)	5.5	5.5	0.0	!meth_s
assign (resid 6 and name ha)	(resid 6 and name hg#)	2.8	2.8	0.0	!dgnm_i
assign (resid 10 and name he#)	(resid 19 and name hn)	3.6	3.6	0.0	!dgnm_l
assign (resid 10 and name he#)	(resid 28 and name hn)	3.6	3.6	0.0	!dgnm_l
assign (resid 14 and name hn)	(resid 14 and name hg#)	3.6	3.6	0.0	!dgnm_i
assign (resid 14 and name hb#)	(resid 15 and name hn)	4.1	4.1	0.0	!dgnm_s
assign (resid 24 and name hn)	(resid 24 and name hg#)	3.6	3.6	0.0	!dgnm_i
assign (resid 24 and name ha)	(resid 24 and name hg#)	2.8	2.8	0.0	!dgnm_i
assign (resid 24 and name hg#)	(resid 25 and name hn)	5.5	5.5	0.0	!dgnm_s
assign (resid 25 and name hn)	(resid 25 and name hd#)	5.5	5.5	0.0	!dgnm_i
assign (resid 26 and name hn)	(resid 26 and name hb#)	2.8	2.8	0.0	!dgnm_i
assign (resid 29 and name hb#)	(resid 29 and name hd#)	3.6	3.6	0.0	!dgnm_i
assign (resid 29 and name hb#)	(resid 30 and name hn)	3.6	3.6	0.0	!dgnm_s
assign (resid 33 and name hn)	(resid 47 and name hg#)	5.5	5.5	0.0	!dgnm_l
assign (resid 35 and name hn)	(resid 35 and name hg#)	3.6	3.6	0.0	!dgnm_i
assign (resid 35 and name ha)	(resid 35 and name hg#)	3.6	3.6	0.0	!dgnm_i
assign (resid 35 and name hg#)	(resid 36 and name hn)	3.6	3.6	0.0	!dgnm_s
assign (resid 35 and name hg#)	(resid 42 and name hn)	5.5	5.5	0.0	!dgnm_l
assign (resid 35 and name hg#)	(resid 44 and name ha)	3.6	3.6	0.0	!dgnm_l
assign (resid 35 and name hg#)	(resid 44 and name hb#)	3.6	3.6	0.0	!dgnm_l
assign (resid 36 and name hn)	(resid 36 and name hg#)	3.6	3.6	0.0	!dgnm_i
assign (resid 36 and name ha)	(resid 36 and name hg#)	2.8	2.8	0.0	!dgnm_i
assign (resid 36 and name hb#)	(resid 37 and name hn)	5.5	5.5	0.0	!dgnm_s
assign (resid 36 and name hg#)	(resid 37 and name hn)	3.6	3.6	0.0	!dgnm_s

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assign (resid 36 and name hg#)	(resid 40 and name hn)	5.5 5.5 0.0	!dgnm_m
assign (resid 37 and name ha)	(resid 37 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 37 and name hg#)	(resid 38 and name hn)	4.1 4.1 0.0	!dgnm_s
assign (resid 40 and name hn)	(resid 40 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 40 and name hn)	(resid 40 and name hd#)	5.5 5.5 0.0	!dgnm_i
assign (resid 44 and name ha)	(resid 45 and name hg#)	3.6 3.6 0.0	!dgnm_s
assign (resid 45 and name hg#)	(resid 46 and name hn)	3.6 3.6 0.0	!dgnm_s
assign (resid 47 and name hg#)	(resid 48 and name hn)	5.5 5.5 0.0	!dgnm_s
assign (resid 49 and name hn)	(resid 51 and name hg#)	3.6 3.6 0.0	!dgnm_m
assign (resid 49 and name hn)	(resid 64 and name hb#)	5.5 5.5 0.0	!dgnm_l
assign (resid 49 and name hn)	(resid 64 and name hg#)	5.5 5.5 0.0	!dgnm_l
assign (resid 51 and name hn)	(resid 51 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 51 and name hg#)	(resid 52 and name hd2#)	5.5 5.5 0.0	!dgnm_s
assign (resid 61 and name hn)	(resid 61 and name hb#)	2.8 2.8 0.0	!dgnm_i
assign (resid 61 and name hb#)	(resid 62 and name hn)	3.6 3.6 0.0	!dgnm_s
assign (resid 64 and name hn)	(resid 64 and name hb#)	2.8 2.8 0.0	!dgnm_i
assign (resid 64 and name hn)	(resid 64 and name hg#)	2.8 2.8 0.0	!dgnm_i
assign (resid 64 and name hn)	(resid 65 and name hg#)	2.8 2.8 0.0	!dgnm_s
assign (resid 64 and name ha)	(resid 64 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 64 and name hb#)	(resid 74 and name ha)	3.6 3.6 0.0	!dgnm_l
assign (resid 65 and name hn)	(resid 65 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 69 and name hb#)	(resid 70 and name hn)	2.8 2.8 0.0	!dgnm_s
assign (resid 71 and name ha#)	(resid 72 and name hn)	2.6 2.6 0.0	!dgnm_s
assign (resid 72 and name hn)	(resid 72 and name hd#)	3.6 3.6 0.0	!dgnm_i
assign (resid 73 and name hn)	(resid 73 and name hb#)	2.8 2.8 0.0	!dgnm_i
assign (resid 73 and name hb#)	(resid 74 and name hn)	3.6 3.6 0.0	!dgnm_s
assign (resid 80 and name hn)	(resid 80 and name hb#)	2.8 2.8 0.0	!dgnm_i
assign (resid 80 and name hb#)	(resid 81 and name hd#)	2.8 2.8 0.0	!dgnm_s
assign (resid 80 and name hb#)	(resid 83 and name hn)	3.6 3.6 0.0	!dgnm_m
assign (resid 80 and name hb#)	(resid 83 and name hb#)	2.8 2.8 0.0	!dgnm_m
assign (resid 84 and name ha)	(resid 85 and name hg#)	3.6 3.6 0.0	!dgnm_s
assign (resid 84 and name ha)	(resid 85 and name hd#)	2.8 2.8 0.0	!dgnm_s
assign (resid 85 and name hg#)	(resid 89 and name hn)	5.5 5.5 0.0	!dgnm_m
assign (resid 86 and name hb#)	(resid 87 and name hn)	3.6 3.6 0.0	!dgnm_s
assign (resid 86 and name hb#)	(resid 91 and name hb#)	3.6 3.6 0.0	!dgnm_l

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assign (resid 9 and name ha)	(resid 8 and name hg#)	3.6 3.6 0.0	!meth_i
assign (resid 9 and name hg#)	(resid 20 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 9 and name hg#)	(resid 20 and name he)	5.5 5.5 0.0	!meth_l
assign (resid 15 and name ha)	(resid 31 and name hd1#)	3.6 3.6 0.0	!meth_l
assign (resid 15 and name hb#)	(resid 31 and name hd1#)	5.5 5.5 0.0	!meth_l
assign (resid 16 and name hn)	(resid 31 and name hd1#)	5.5 5.5 0.0	!meth_l
assign (resid 16 and name ha)	(resid 31 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 17 and name hn)	(resid 31 and name hd2#)	4.1 4.1 0.0	!meth_l
assign (resid 17 and name ha#)	(resid 31 and name hd2#)	5.5 5.5 0.0	!meth_l
assign (resid 19 and name hz)	(resid 22 and name hd#)	3.6 3.6 0.0	!meth_m
assign (resid 22 and name hd#)	(resid 23 and name hn)	5.5 5.5 0.0	!meth_s
assign (resid 30 and name ha)	(resid 31 and name hd#)	5.5 5.5 0.0	!meth_s
assign (resid 31 and name ha)	(resid 31 and name hd2#)	3.6 3.6 0.0	!meth_i
assign (resid 31 and name hd2#)	(resid 87 and name hb#)	5.5 5.5 0.0	!meth_l
assign (resid 31 and name hd2#)	(resid 87 and name hz)	3.6 3.6 0.0	!meth_l
assign (resid 31 and name hd2#)	(resid 90 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 31 and name hd2#)	(resid 90 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name ha)	(resid 32 and name hd2#)	3.6 3.6 0.0	!meth_i
assign (resid 32 and name hd2#)	(resid 33 and name hn)	3.6 3.6 0.0	!meth_s
assign (resid 32 and name hd2#)	(resid 55 and name ha#)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name hd2#)	(resid 56 and name hn)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name hd2#)	(resid 56 and name ha)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name hd2#)	(resid 74 and name hb)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name hd2#)	(resid 74 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name hd2#)	(resid 90 and name ha)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name hd2#)	(resid 90 and name hg2#)	3.6 3.6 0.0	!meth_l
assign (resid 32 and name hd1#)	(resid 88 and name hb#)	5.5 5.5 0.0	!meth_l

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assign (resid 35 and name hn)	(resid 42 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 35 and name hb2)	(resid 42 and name hg1#)	5.5	5.5	0.0	!meth_l
assign (resid 35 and name hg#)	(resid 42 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 37 and name hn)	(resid 42 and name hg1#)	5.5	5.5	0.0	!meth_l
assign (resid 37 and name hn)	(resid 42 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 37 and name ha)	(resid 42 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 37 and name hb#)	(resid 42 and name hg1#)	5.5	5.5	0.0	!meth_l
assign (resid 37 and name hb#)	(resid 42 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 37 and name hg#)	(resid 42 and name hg1#)	5.5	5.5	0.0	!meth_l
assign (resid 37 and name hg#)	(resid 42 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 40 and name hn)	(resid 42 and name hg1#)	5.5	5.5	0.0	!meth_m
assign (resid 40 and name hg#)	(resid 42 and name hg1#)	5.5	5.5	0.0	!meth_m
assign (resid 41 and name ha)	(resid 42 and name hg1#)	5.5	5.5	0.0	!meth_s
assign (resid 41 and name ha)	(resid 42 and name hg2#)	5.5	5.5	0.0	!meth_s
assign (resid 41 and name hb#)	(resid 42 and name hg#)	5.5	5.5	0.0	!meth_s
assign (resid 42 and name hg1#)	(resid 43 and name hn)	3.6	3.6	0.0	!meth_s
assign (resid 42 and name hg2#)	(resid 43 and name hn)	3.6	3.6	0.0	!meth_s
assign (resid 5 and name ha)	(resid 20 and name hb#)	3.6	3.6	0.0	!nsam_l
assign (resid 7 and name ha)	(resid 20 and name hb#)	3.6	3.6	0.0	!nsam_l
assign (resid 7 and name ha)	(resid 20 and name hd#)	3.6	3.6	0.0	!nsam_l
assign (resid 10 and name ha)	(resid 10 and name hg#)	3.6	3.6	0.0	!nsam_i
assign (resid 12 and name ha)	(resid 13 and name hd#)	2.8	2.8	0.0	!nsam_s
assign (resid 12 and name hb#)	(resid 13 and name hd#)	3.6	3.6	0.0	!nsam_s
assign (resid 12 and name hb#)	(resid 28 and name hb#)	3.6	3.6	0.0	!nsam_l
assign (resid 13 and name hb2)	(resid 14 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 13 and name hg#)	(resid 16 and name hb#)	3.6	3.6	0.0	!nsam_m
assign (resid 13 and name hd#)	(resid 28 and name hb#)	3.6	3.6	0.0	!nsam_l
assign (resid 15 and name hb2)	(resid 41 and name hb2)	3.6	3.6	0.0	!sing_l
assign (resid 16 and name ha)	(resid 30 and name hb#)	3.6	3.6	0.0	!nsam_l
assign (resid 16 and name hb#)	(resid 30 and name ha)	3.6	3.6	0.0	!nsam_l
assign (resid 16 and name hb#)	(resid 31 and name hn)	3.6	3.6	0.0	!nsam_l
assign (resid 17 and name ha#)	(resid 87 and name hz)	3.6	3.6	0.0	!nsam_l
assign (resid 20 and name ha)	(resid 20 and name hg#)	3.6	3.6	0.0	!nsam_i
assign (resid 20 and name ha)	(resid 26 and name hg#)	3.6	3.6	0.0	!nsam_l
assign (resid 20 and name hb#)	(resid 20 and name hd#)	3.6	3.6	0.0	!nsam_i
assign (resid 20 and name hg#)	(resid 21 and name hn)	3.6	3.6	0.0	!nsam_s
assign (resid 20 and name hg#)	(resid 24 and name ha)	3.6	3.6	0.0	!nsam_m
assign (resid 21 and name hb#)	(resid 22 and name hn)	5.5	5.5	0.0	!nsam_s
assign (resid 21 and name hb#)	(resid 23 and name hn)	5.5	5.5	0.0	!nsam_m
assign (resid 22 and name hb2)	(resid 23 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 24 and name hb#)	(resid 25 and name hn)	5.5	5.5	0.0	!nsam_s
assign (resid 25 and name hn)	(resid 25 and name hg#)	3.6	3.6	0.0	!nsam_i
assign (resid 25 and name hg#)	(resid 26 and name hn)	3.6	3.6	0.0	!nsam_s
assign (resid 26 and name hn)	(resid 26 and name hg#)	3.6	3.6	0.0	!nsam_i
assign (resid 26 and name ha)	(resid 26 and name hg#)	3.6	3.6	0.0	!nsam_i
assign (resid 27 and name ha)	(resid 27 and name hg#)	2.8	2.8	0.0	!nsam_i
assign (resid 27 and name hb#)	(resid 28 and name hn)	2.8	2.8	0.0	!nsam_s
assign (resid 27 and name hg#)	(resid 29 and name hn)	2.8	2.8	0.0	!nsam_s
assign (resid 28 and name hn)	(resid 29 and name hb#)	2.8	2.8	0.0	!nsam_i
assign (resid 29 and name hg#)	(resid 30 and name hn)	2.8	2.8	0.0	!nsam_s
assign (resid 29 and name hd#)	(resid 90 and name hb)	3.6	3.6	0.0	!nsam_l
assign (resid 30 and name hn)	(resid 30 and name hb#)	2.8	2.8	0.0	!nsam_i
assign (resid 30 and name hb1)	(resid 31 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 30 and name hb#)	(resid 34 and name hn)	5.5	5.5	0.0	!nsam_m
assign (resid 30 and name hb2)	(resid 34 and name hb2)	3.6	3.6	0.0	!sing_m
assign (resid 30 and name hb#)	(resid 35 and name ha)	3.6	3.6	0.0	!nsam_l
assign (resid 31 and name hn)	(resid 31 and name hb#)	3.6	3.6	0.0	!nsam_i
assign (resid 31 and name hn)	(resid 34 and name hb2)	3.6	3.6	0.0	!sing_m
assign (resid 32 and name hb1)	(resid 33 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 33 and name ha)	(resid 44 and name hb#)	3.6	3.6	0.0	!nsam_l
assign (resid 33 and name ha)	(resid 47 and name hb1)	3.6	3.6	0.0	!sing_l
assign (resid 33 and name hb2)	(resid 34 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 34 and name ha)	(resid 44 and name hb#)	3.6	3.6	0.0	!nsam_l
assign (resid 34 and name hb1)	(resid 35 and name hn)	4.1	4.1	0.0	!sing_s
assign (resid 35 and name hb#)	(resid 35 and name hd#)	2.8	2.8	0.0	!nsam_i

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assign (resid 35 and name hb2)	(resid 36 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 35 and name hb#)	(resid 44 and name hb#)	3.6	3.6	0.0	!nsam_l
assign (resid 35 and name hd#)	(resid 44 and name ha)	3.6	3.6	0.0	!nsam_l
assign (resid 37 and name hn)	(resid 40 and name hb#)	3.6	3.6	0.0	!nsam_m
assign (resid 38 and name ha#)	(resid 39 and name hn)	3.1	3.1	0.0	!nsam_s
assign (resid 38 and name ha#)	(resid 40 and name hn)	3.6	3.6	0.0	!nsam_m
assign (resid 39 and name hb#)	(resid 40 and name hn)	3.6	3.6	0.0	!nsam_s
assign (resid 41 and name hb1)	(resid 42 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 43 and name hn)	(resid 43 and name hb#)	2.8	2.8	0.0	!nsam_i
assign (resid 43 and name ha)	(resid 43 and name hg#)	2.8	2.8	0.0	!nsam_i
assign (resid 43 and name hb#)	(resid 44 and name hn)	3.6	3.6	0.0	!nsam_s
assign (resid 43 and name hg#)	(resid 44 and name hn)	3.6	3.6	0.0	!nsam_s
assign (resid 44 and name hn)	(resid 45 and name hd#)	5.5	5.5	0.0	!nsam_s
assign (resid 44 and name ha)	(resid 45 and name hd#)	2.8	2.8	0.0	!nsam_s
assign (resid 45 and name hb2)	(resid 46 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 46 and name hn)	(resid 46 and name hb#)	3.6	3.6	0.0	!nsam_i
assign (resid 46 and name hn)	(resid 47 and name hd#)	3.6	3.6	0.0	!nsam_s
assign (resid 46 and name ha)	(resid 47 and name hd#)	3.6	3.6	0.0	!nsam_s
assign (resid 47 and name hb2)	(resid 48 and name hn)	4.1	4.1	0.0	!sing_s
assign (resid 47 and name hb2)	(resid 74 and name hgl#)	3.6	3.6	0.0	!nsam_l
assign (resid 47 and name hg#)	(resid 74 and name hgl#)	3.6	3.6	0.0	!nsam_l
assign (resid 48 and name hn)	(resid 54 and name ha#)	4.1	4.1	0.0	!nsam_l
assign (resid 48 and name hn)	(resid 74 and name hgl#)	5.5	5.5	0.0	!nsam_l
assign (resid 49 and name ha)	(resid 54 and name ha#)	3.6	3.6	0.0	!nsam_l
assign (resid 49 and name ha)	(resid 62 and name hb1)	5.5	5.5	0.0	!sing_l
assign (resid 50 and name hb1)	(resid 51 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 53 and name hb#)	(resid 56 and name hn)	5.5	5.5	0.0	!nsam_m
assign (resid 53 and name hb1)	(resid 58 and name ha)	3.6	3.6	0.0	!sing_l
assign (resid 53 and name hd22)	(resid 56 and name hn)	5.5	5.5	0.0	!sing_m
assign (resid 53 and name hd21)	(resid 60 and name hn)	5.5	5.5	0.0	!sing_l
assign (resid 54 and name ha#)	(resid 55 and name hn)	3.6	3.6	0.0	!nsam_s
assign (resid 54 and name ha#)	(resid 56 and name hn)	3.6	3.6	0.0	!nsam_m
assign (resid 55 and name ha#)	(resid 56 and name hn)	3.6	3.6	0.0	!nsam_s
assign (resid 56 and name hb1)	(resid 57 and name hn)	4.1	4.1	0.0	!sing_s
assign (resid 56 and name hb2)	(resid 62 and name hn)	5.5	5.5	0.0	!sing_l
assign (resid 56 and name hb2)	(resid 62 and name ha)	3.6	3.6	0.0	!sing_l
assign (resid 57 and name hb#)	(resid 58 and name hn)	3.6	3.6	0.0	!nsam_s
assign (resid 57 and name hb#)	(resid 59 and name hn)	4.1	4.1	0.0	!nsam_m
assign (resid 57 and name hb#)	(resid 60 and name hn)	4.1	4.1	0.0	!nsam_m
assign (resid 57 and name hb#)	(resid 91 and name ha)	2.8	2.8	0.0	!nsam_l
assign (resid 57 and name hb#)	(resid 92 and name hn)	5.5	5.5	0.0	!nsam_l
assign (resid 58 and name hn)	(resid 59 and name hb#)	5.5	5.5	0.0	!nsam_s
assign (resid 59 and name hb#)	(resid 80 and name hg#)	3.6	3.6	0.0	!nsam_l
assign (resid 59 and name hb#)	(resid 60 and name hn)	3.6	3.6	0.0	!nsam_s
assign (resid 60 and name ha)	(resid 78 and name hb#)	3.6	3.6	0.0	!nsam_l
assign (resid 61 and name hn)	(resid 61 and name hg#)	3.6	3.6	0.0	!nsam_i
assign (resid 65 and name hn)	(resid 65 and name hb#)	2.8	2.8	0.0	!nsam_i
assign (resid 66 and name hn)	(resid 66 and name hb#)	2.8	2.8	0.0	!nsam_i
assign (resid 66 and name hb#)	(resid 67 and name hn)	3.6	3.6	0.0	!nsam_s
assign (resid 67 and name hb#)	(resid 73 and name hb#)	3.6	3.6	0.0	!nsam_l
assign (resid 68 and name ha#)	(resid 69 and name hn)	3.6	3.6	0.0	!nsam_s
assign (resid 72 and name hn)	(resid 72 and name hg#)	3.6	3.6	0.0	!nsam_i
assign (resid 74 and name hn)	(resid 74 and name hgl#)	3.6	3.6	0.0	!nsam_i
assign (resid 74 and name ha)	(resid 74 and name hgl#)	3.6	3.6	0.0	!nsam_i
assign (resid 76 and name hn)	(resid 76 and name hb#)	2.8	2.8	0.0	!nsam_i
assign (resid 76 and name hb1)	(resid 77 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 76 and name hb1)	(resid 39 and name ha#)	3.6	3.6	0.0	!nsam_l
assign (resid 73 and name hb1)	(resid 79 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 78 and name hb#)	(resid 92 and name hb#)	3.6	3.6	0.0	!nsam_l
assign (resid 78 and name hb#)	(resid 80 and name hn)	5.5	5.5	0.0	!nsam_m
assign (resid 78 and name hb#)	(resid 83 and name hb#)	3.6	3.6	0.0	!nsam_l
assign (resid 80 and name hn)	(resid 80 and name hg#)	3.6	3.6	0.0	!nsam_i
assign (resid 80 and name hn)	(resid 81 and name hd#)	5.5	5.5	0.0	!nsam_s
assign (resid 80 and name hn)	(resid 83 and name hb#)	3.6	3.6	0.0	!nsam_m
assign (resid 80 and name ha)	(resid 80 and name hg#)	3.6	3.6	0.0	!nsam_i

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assign (resid 80 and name ha)	(resid 81 and name hd#)	2.8	2.8	0.0	!nsam_s
assign (resid 80 and name hg#)	(resid 81 and name hd#)	3.6	3.6	0.0	!nsam_s
assign (resid 81 and name hb2)	(resid 82 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 81 and name hd#)	(resid 82 and name hn)	5.5	5.5	0.0	!nsam_s
assign (resid 82 and name hb#)	(resid 83 and name hn)	3.6	3.6	0.0	!nsam_s
assign (resid 83 and name ha)	(resid 89 and name ha#)	3.6	3.6	0.0	!nsam_m
assign (resid 85 and name hb#)	(resid 89 and name ha)	3.6	3.6	0.0	!nsam_m
assign (resid 85 and name hb#)	(resid 89 and name hn)	3.6	3.6	0.0	!nsam_m
assign (resid 85 and name hb#)	(resid 86 and name hn)	3.6	3.6	0.0	!nsam_s
assign (resid 86 and name hn)	(resid 89 and name ha#)	5.5	5.5	0.0	!nsam_m
assign (resid 87 and name hb#)	(resid 90 and name hn)	3.6	3.6	0.0	!nsam_m
assign (resid 87 and name hb#)	(resid 90 and name hg1#)	5.5	5.5	0.0	!nsam_m
assign (resid 89 and name ha#)	(resid 90 and name hn)	3.6	3.6	0.0	!nsam_s
assign (resid 90 and name hg1#)	(resid 91 and name hn)	4.1	4.1	0.0	!nsam_s

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assign (resid 2 and name hn)	(resid 3 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 4 and name hn)	(resid 5 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 5 and name hn)	(resid 6 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 5 and name ha)	(resid 20 and name hn)	5.5	5.5	0.0	!sing_l
assign (resid 6 and name hn)	(resid 7 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 7 and name hn)	(resid 8 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 8 and name hn)	(resid 9 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 9 and name hn)	(resid 10 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 13 and name ha)	(resid 14 and name hn)	2.6	2.6	0.0	!sing_s
assign (resid 14 and name hn)	(resid 15 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 14 and name ha)	(resid 15 and name hn)	2.6	2.6	0.0	!sing_m
assign (resid 15 and name hn)	(resid 16 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 15 and name ha)	(resid 16 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 15 and name ha)	(resid 31 and name hg)	3.6	3.6	0.0	!sing_l
assign (resid 16 and name hn)	(resid 17 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 16 and name hn)	(resid 31 and name hn)	5.5	5.5	0.0	!sing_l
assign (resid 16 and name hn)	(resid 31 and name hg)	5.5	5.5	0.0	!sing_l
assign (resid 16 and name ha)	(resid 30 and name ha)	2.8	2.8	0.0	!sing_l
assign (resid 16 and name ha)	(resid 31 and name hn)	3.6	3.6	0.0	!sing_l
assign (resid 16 and name ha)	(resid 31 and name hg)	3.6	3.6	0.0	!sing_l
assign (resid 17 and name hn)	(resid 31 and name hn)	5.5	5.5	0.0	!sing_l
assign (resid 18 and name ha)	(resid 19 and name hn)	2.6	2.6	0.0	!sing_s
assign (resid 18 and name ha)	(resid 28 and name ha)	2.8	2.8	0.0	!sing_l
assign (resid 19 and name hn)	(resid 20 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 19 and name hn)	(resid 21 and name hd2)	5.5	5.5	0.0	!sing_m
assign (resid 19 and name hn)	(resid 28 and name ha)	5.5	5.5	0.0	!sing_l
assign (resid 19 and name ha)	(resid 20 and name hn)	3.1	3.1	0.0	!sing_s
assign (resid 19 and name hz)	(resid 60 and name hn)	5.5	5.5	0.0	!sing_l
assign (resid 20 and name ha)	(resid 21 and name hn)	3.1	3.1	0.0	!sing_s
assign (resid 20 and name ha)	(resid 26 and name ha)	3.6	3.6	0.0	!sing_l
assign (resid 21 and name hn)	(resid 21 and name hd2)	3.6	3.6	0.0	!sing_i
assign (resid 21 and name hn)	(resid 25 and name hn)	5.5	5.5	0.0	!sing_m
assign (resid 21 and name ha)	(resid 22 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 21 and name ha)	(resid 23 and name hn)	3.6	3.6	0.0	!sing_m
assign (resid 22 and name hn)	(resid 22 and name hg)	5.5	5.5	0.0	!sing_i
assign (resid 22 and name hn)	(resid 23 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 22 and name hn)	(resid 25 and name hn)	5.5	5.5	0.0	!sing_m
assign (resid 22 and name ha)	(resid 23 and name hn)	4.1	4.1	0.0	!sing_s
assign (resid 22 and name ha)	(resid 24 and name hn)	4.1	4.1	0.0	!sing_m
assign (resid 22 and name hg)	(resid 23 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 23 and name hn)	(resid 24 and name hn)	4.1	4.1	0.0	!sing_s
assign (resid 23 and name hn)	(resid 24 and name ha)	5.5	5.5	0.0	!sing_s
assign (resid 23 and name ha)	(resid 24 and name hn)	4.1	4.1	0.0	!sing_s
assign (resid 23 and name ha)	(resid 25 and name hn)	5.5	5.5	0.0	!sing_m
assign (resid 24 and name ha)	(resid 25 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 25 and name hn)	(resid 26 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 25 and name ha)	(resid 26 and name hn)	2.6	2.6	0.0	!sing_s
assign (resid 26 and name hn)	(resid 27 and name hn)	5.5	5.5	0.0	!sing_s

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assign (resid 26 and name ha)	(resid 27 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 27 and name ha)	(resid 28 and name hn)	2.6	2.6	0.0	!sing_s
assign (resid 28 and name ha)	(resid 29 and name hn)	3.1	3.1	0.0	!sing_s
assign (resid 29 and name ha)	(resid 30 and name hn)	2.6	2.6	0.0	!sing_s
assign (resid 30 and name ha)	(resid 31 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 30 and name ha)	(resid 31 and name hn)	3.1	3.1	0.0	!sing_s
assign (resid 31 and name hn)	(resid 31 and name hg)	3.6	3.6	0.0	!sing_i
assign (resid 31 and name hn)	(resid 32 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 31 and name ha)	(resid 32 and name hn)	3.1	3.1	0.0	!sing_s
assign (resid 31 and name ha)	(resid 31 and name hg)	3.6	3.6	0.0	!sing_i
assign (resid 32 and name ha)	(resid 32 and name hg)	3.6	3.6	0.0	!sing_i
assign (resid 32 and name ha)	(resid 33 and name hn)	2.6	2.6	0.0	!sing_s
assign (resid 32 and name ha)	(resid 34 and name hn)	3.6	3.6	0.0	!sing_m
assign (resid 33 and name hn)	(resid 34 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 33 and name ha)	(resid 34 and name hn)	3.1	3.1	0.0	!sing_s
assign (resid 34 and name hn)	(resid 35 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 34 and name hn)	(resid 44 and name hn)	5.5	5.5	0.0	!sing_l
assign (resid 34 and name ha)	(resid 35 and name hn)	3.1	3.1	0.0	!sing_s
assign (resid 34 and name ha)	(resid 43 and name ha)	2.8	2.8	0.0	!sing_l
assign (resid 34 and name ha)	(resid 44 and name hn)	3.1	3.1	0.0	!sing_l
assign (resid 35 and name hn)	(resid 36 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 35 and name hn)	(resid 41 and name ha)	5.5	5.5	0.0	!sing_l
assign (resid 35 and name hn)	(resid 42 and name hn)	3.6	3.6	0.0	!sing_l
assign (resid 35 and name hn)	(resid 43 and name ha)	5.5	5.5	0.0	!sing_l
assign (resid 35 and name hn)	(resid 44 and name hn)	5.5	5.5	0.0	!sing_l
assign (resid 35 and name hn)	(resid 44 and name ha)	5.5	5.5	0.0	!sing_l
assign (resid 35 and name ha)	(resid 36 and name hn)	2.6	2.6	0.0	!sing_s
assign (resid 36 and name hn)	(resid 37 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 36 and name ha)	(resid 37 and name hn)	2.6	2.6	0.0	!sing_s
assign (resid 36 and name ha)	(resid 41 and name ha)	2.8	2.8	0.0	!sing_l
assign (resid 36 and name ha)	(resid 42 and name hn)	3.6	3.6	0.0	!sing_l
assign (resid 37 and name hn)	(resid 40 and name hn)	3.6	3.6	0.0	!sing_m
assign (resid 37 and name hn)	(resid 41 and name ha)	3.5	3.6	0.0	!sing_m
assign (resid 37 and name hn)	(resid 42 and name hn)	5.5	5.5	0.0	!sing_l
assign (resid 37 and name ha)	(resid 38 and name hn)	2.6	2.6	0.0	!sing_s
assign (resid 38 and name hn)	(resid 39 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 39 and name hn)	(resid 40 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 40 and name hn)	(resid 41 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 40 and name ha)	(resid 41 and name hn)	2.6	2.6	0.0	!sing_s
assign (resid 41 and name hn)	(resid 42 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 41 and name ha)	(resid 42 and name hn)	2.6	2.6	0.0	!sing_s
assign (resid 42 and name ha)	(resid 43 and name hn)	2.6	2.6	0.0	!sing_s
assign (resid 42 and name hb)	(resid 43 and name hn)	3.1	3.1	0.0	!sing_s
assign (resid 43 and name hn)	(resid 44 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 43 and name ha)	(resid 44 and name hn)	2.6	2.6	0.0	!sing_s
assign (resid 44 and name ha)	(resid 46 and name hn)	3.6	3.6	0.0	!sing_m
assign (resid 45 and name ha)	(resid 46 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 46 and name ha)	(resid 72 and name hn)	5.5	5.5	0.0	!sing_l
assign (resid 47 and name ha)	(resid 48 and name hn)	2.6	2.6	0.0	!sing_s
assign (resid 48 and name hn)	(resid 49 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 48 and name hn)	(resid 51 and name hn)	5.5	5.5	0.0	!sing_m
assign (resid 48 and name ha)	(resid 49 and name hn)	3.1	3.1	0.0	!sing_s
assign (resid 48 and name ha)	(resid 50 and name hn)	5.5	5.5	0.0	!sing_m
assign (resid 48 and name hb)	(resid 49 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 48 and name hb)	(resid 50 and name hn)	5.5	5.5	0.0	!sing_m
assign (resid 49 and name hn)	(resid 50 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 49 and name hn)	(resid 51 and name hn)	5.5	5.5	0.0	!sing_m
assign (resid 49 and name ha)	(resid 50 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 49 and name ha)	(resid 51 and name hn)	4.1	4.1	0.0	!sing_m
assign (resid 49 and name ha)	(resid 53 and name ha)	3.6	3.6	0.0	!sing_m
assign (resid 49 and name ha)	(resid 54 and name hn)	3.6	3.6	0.0	!sing_l
assign (resid 50 and name hn)	(resid 51 and name hn)	3.6	3.6	0.0	!sing_s
assign (resid 51 and name hn)	(resid 52 and name hn)	5.5	5.5	0.0	!sing_s
assign (resid 51 and name hn)	(resid 53 and name hn)	4.1	4.1	0.0	!sing_m
assign (resid 51 and name hn)	(resid 54 and name hn)	4.1	4.1	0.0	!sing_m

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assign (resid 51 and name ha)	(resid 52 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 52 and name hn)	(resid 53 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 52 and name ha)	(resid 53 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 53 and name hn)	(resid 54 and name hn)	4.1 4.1 0.0	!sing_s
assign (resid 53 and name ha)	(resid 54 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 53 and name hn)	(resid 56 and name hn)	2.8 2.8 0.0	!sing_m
assign (resid 54 and name hn)	(resid 55 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 55 and name hn)	(resid 56 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 56 and name hn)	(resid 57 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 56 and name ha)	(resid 57 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 57 and name hn)	(resid 59 and name hn)	5.5 5.5 0.0	!sing_m
assign (resid 57 and name hn)	(resid 60 and name hn)	5.5 5.5 0.0	!sing_m
assign (resid 57 and name hn)	(resid 90 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 57 and name hn)	(resid 90 and name ha)	2.6 2.6 0.0	!sing_l
assign (resid 57 and name hn)	(resid 91 and name ha)	5.5 5.5 0.0	!sing_l
assign (resid 57 and name hn)	(resid 91 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 57 and name ha)	(resid 91 and name ha)	3.6 3.6 0.0	!sing_l
assign (resid 58 and name hn)	(resid 59 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 58 and name hn)	(resid 60 and name hn)	5.5 5.5 0.0	!sing_m
assign (resid 58 and name ha)	(resid 59 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 59 and name hn)	(resid 60 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 59 and name ha)	(resid 60 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 60 and name hn)	(resid 61 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 60 and name hn)	(resid 78 and name ha)	5.5 5.5 0.0	!sing_l
assign (resid 60 and name ha)	(resid 61 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 60 and name ha)	(resid 77 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 60 and name ha)	(resid 78 and name ha)	2.8 2.8 0.0	!sing_l
assign (resid 60 and name ha)	(resid 79 and name hn)	3.6 3.6 0.0	!sing_l
assign (resid 61 and name hn)	(resid 77 and name hn)	3.6 3.6 0.0	!sing_l
assign (resid 61 and name hn)	(resid 78 and name ha)	3.6 3.6 0.0	!sing_l
assign (resid 61 and name hn)	(resid 79 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 61 and name ha)	(resid 62 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 62 and name ha)	(resid 63 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 62 and name ha)	(resid 76 and name ha)	2.8 2.8 0.0	!sing_l
assign (resid 63 and name hn)	(resid 63 and name hb)	3.6 3.6 0.0	!sing_i
assign (resid 63 and name hn)	(resid 64 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 63 and name hn)	(resid 75 and name hn)	3.6 3.6 0.0	!sing_l
assign (resid 63 and name hn)	(resid 76 and name ha)	5.5 5.5 0.0	!sing_l
assign (resid 63 and name ha)	(resid 64 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 63 and name hb)	(resid 64 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 64 and name ha)	(resid 74 and name ha)	2.8 2.8 0.0	!sing_l
assign (resid 65 and name ha)	(resid 66 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 66 and name hn)	(resid 67 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 67 and name hn)	(resid 68 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 67 and name ha)	(resid 68 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 70 and name hn)	(resid 71 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 70 and name ha)	(resid 71 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 71 and name hn)	(resid 72 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 72 and name hn)	(resid 73 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 72 and name ha)	(resid 73 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 73 and name hn)	(resid 74 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 73 and name ha)	(resid 74 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 74 and name hn)	(resid 74 and name hb)	3.1 3.1 0.0	!sing_i
assign (resid 74 and name ha)	(resid 75 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 75 and name hn)	(resid 75 and name hb)	3.1 3.1 0.0	!sing_i
assign (resid 75 and name ha)	(resid 76 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 75 and name hb)	(resid 76 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 76 and name ha)	(resid 77 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 77 and name hn)	(resid 78 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 77 and name ha)	(resid 78 and name hn)	2.8 2.8 0.0	!sing_s
assign (resid 78 and name hn)	(resid 79 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 78 and name ha)	(resid 79 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 79 and name hn)	(resid 79 and name hb)	3.6 3.6 0.0	!sing_i
assign (resid 79 and name hn)	(resid 80 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 79 and name hb)	(resid 80 and name hn)	5.5 5.5 0.0	!sing_s

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assign (resid 81 and name ha) (resid 82 and name hn) 3.6 3.6 0.0 !sing_s
assign (resid 81 and name ha) (resid 83 and name hn) 4.1 4.1 0.0 !sing_m
assign (resid 82 and name hn) (resid 83 and name hn) 4.1 4.1 0.0 !sing_s
assign (resid 82 and name ha) (resid 83 and name hn) 4.1 4.1 0.0 !sing_s
assign (resid 83 and name hn) (resid 84 and name hn) 5.5 5.5 0.0 !sing_s
assign (resid 83 and name ha) (resid 84 and name hn) 2.6 2.6 0.0 !sing_s
assign (resid 84 and name hn) (resid 92 and name hn) 5.5 5.5 0.0 !sing_i
assign (resid 84 and name hn) (resid 93 and name hn) 5.5 5.5 0.0 !sing_i
assign (resid 85 and name ha) (resid 86 and name hn) 2.6 2.6 0.0 !sing_s
assign (resid 85 and name ha) (resid 92 and name ha) 2.8 2.8 0.0 !sing_i
assign (resid 86 and name hn) (resid 86 and name hg) 3.6 3.6 0.0 !sing_i
assign (resid 86 and name hn) (resid 87 and name hn) 5.5 5.5 0.0 !sing_s
assign (resid 86 and name hn) (resid 89 and name hn) 4.1 4.1 0.0 !sing_m
assign (resid 86 and name hn) (resid 90 and name hn) 5.5 5.5 0.0 !sing_m
assign (resid 86 and name hn) (resid 91 and name hn) 3.6 3.6 0.0 !sing_l
assign (resid 86 and name ha) (resid 87 and name hn) 3.6 3.6 0.0 !sing_s
assign (resid 87 and name hn) (resid 88 and name hn) 4.1 4.1 0.0 !sing_s
assign (resid 87 and name ha) (resid 88 and name hn) 3.1 3.1 0.0 !sing_s
assign (resid 88 and name hn) (resid 89 and name hn) 4.1 4.1 0.0 !sing_s
assign (resid 88 and name hn) (resid 90 and name hn) 5.5 5.5 0.0 !sing_m
assign (resid 88 and name ha) (resid 89 and name hn) 4.1 4.1 0.0 !sing_s
assign (resid 89 and name hn) (resid 90 and name hn) 3.6 3.6 0.0 !sing_s
assign (resid 90 and name hn) (resid 90 and name hb) 3.6 3.6 0.0 !sing_i
assign (resid 90 and name hn) (resid 91 and name hn) 3.1 3.1 0.0 !sing_s
assign (resid 90 and name hb) (resid 91 and name hn) 5.5 5.5 0.0 !sing_s
assign (resid 91 and name hn) (resid 92 and name hn) 5.5 5.5 0.0 !sing_s
assign (resid 91 and name ha) (resid 92 and name hn) 3.1 3.1 0.0 !sing_s
assign (resid 92 and name ha) (resid 93 and name hn) 3.1 3.1 0.0 !sing_s

```

class hbnd

```

assign (resid 17 and name hn) (resid 29 and name o) 2.0 0.3 0.3 !hbnd
assign (resid 17 and name n) (resid 29 and name o) 3.3 0.3 0.3 !hbnd
assign (resid 17 and name o) (resid 29 and name hn) 2.0 0.3 0.3 !hbnd
assign (resid 17 and name o) (resid 29 and name n) 3.3 0.3 0.3 !hbnd
assign (resid 19 and name hn) (resid 27 and name o) 2.0 0.3 0.3 !hbnd
assign (resid 19 and name n) (resid 27 and name o) 3.3 0.3 0.3 !hbnd
assign (resid 19 and name o) (resid 27 and name hn) 2.0 0.3 0.3 !hbnd
assign (resid 19 and name o) (resid 27 and name n) 3.3 0.3 0.3 !hbnd
assign (resid 35 and name hn) (resid 42 and name o) 2.0 0.3 0.3 !hbnd
assign (resid 35 and name n) (resid 42 and name o) 3.3 0.3 0.3 !hbnd
assign (resid 35 and name o) (resid 42 and name hn) 2.0 0.3 0.3 !hbnd
assign (resid 35 and name o) (resid 42 and name n) 3.3 0.3 0.3 !hbnd
assign (resid 61 and name hn) (resid 77 and name o) 2.0 0.3 0.3 !hbnd
assign (resid 61 and name n) (resid 77 and name o) 3.3 0.3 0.3 !hbnd
assign (resid 61 and name o) (resid 77 and name hn) 2.0 0.3 0.3 !hbnd
assign (resid 61 and name o) (resid 77 and name n) 3.3 0.3 0.3 !hbnd
assign (resid 63 and name hn) (resid 75 and name o) 2.0 0.3 0.3 !hbnd
assign (resid 63 and name n) (resid 75 and name o) 3.3 0.3 0.3 !hbnd
assign (resid 63 and name o) (resid 75 and name hn) 2.0 0.3 0.3 !hbnd
assign (resid 63 and name o) (resid 75 and name n) 3.3 0.3 0.3 !hbnd

```

! dihedral angle restraints X-PLOR format

```

! chi-1      22 restraints
! phi        25 restraints
! psi        33 restraints
! chi-2       5 restraints

```

!&lt;ENERGY&gt;

&lt;ANGLE&gt; &lt;RANGE&gt;&lt;EXPONENT&gt;

! chi-1 restraints

```

assign (resid 15 and name n) (resid 15 and name ca)
      (resid 15 and name cb) (resid 15 and name cg) 1.0 -60.0 40.0 2

```



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```

assign (resid 19 and name n ) (resid 19 and name ca)
      (resid 19 and name cb) (resid 19 and name cg) 1.0 180.0 40.0 2
assign (resid 22 and name n ) (resid 22 and name ca)
      (resid 22 and name cb) (resid 22 and name cg) 1.0 -60.0 40.0 2
assign (resid 23 and name n ) (resid 23 and name ca)
      (resid 23 and name cb) (resid 23 and name cg) 1.0 60.0 40.0 2
assign (resid 30 and name n ) (resid 30 and name ca)
      (resid 30 and name cb) (resid 30 and name sg) 1.0 -60.0 40.0 2
assign (resid 31 and name n ) (resid 31 and name ca)
      (resid 31 and name cb) (resid 31 and name cg) 1.0 -60.0 40.0 2
assign (resid 32 and name n ) (resid 32 and name ca)
      (resid 32 and name cb) (resid 32 and name cg) 1.0 -60.0 40.0 2
assign (resid 33 and name n ) (resid 33 and name ca)
      (resid 33 and name cb) (resid 33 and name cg) 1.0 -60.0 40.0 2
assign (resid 34 and name n ) (resid 34 and name ca)
      (resid 34 and name cb) (resid 34 and name cg) 1.0 -60.0 40.0 2
assign (resid 35 and name n ) (resid 35 and name ca)
      (resid 35 and name cb) (resid 35 and name cg) 1.0 60.0 40.0 2
assign (resid 41 and name n ) (resid 41 and name ca)
      (resid 41 and name cb) (resid 41 and name sg) 1.0 -60.0 40.0 2
assign (resid 42 and name n ) (resid 42 and name ca)
      (resid 42 and name cb) (resid 42 and name cg1) 1.0 -60.0 40.0 2
assign (resid 49 and name n ) (resid 49 and name ca)
      (resid 49 and name cb) (resid 49 and name sg) 1.0 -60.0 40.0 2
assign (resid 52 and name n ) (resid 52 and name ca)
      (resid 52 and name cb) (resid 52 and name cg) 1.0 60.0 40.0 2
assign (resid 53 and name n ) (resid 53 and name ca)
      (resid 53 and name cb) (resid 53 and name cg) 1.0 180.0 40.0 2
assign (resid 56 and name n ) (resid 56 and name ca)
      (resid 56 and name cb) (resid 56 and name sg) 1.0 -60.0 40.0 2
assign (resid 62 and name n ) (resid 62 and name ca)
      (resid 62 and name cb) (resid 62 and name sg) 1.0 180.0 40.0 2
assign (resid 74 and name n ) (resid 74 and name ca)
      (resid 74 and name cb) (resid 74 and name cg1) 1.0 -60.0 40.0 2
assign (resid 76 and name n ) (resid 76 and name ca)
      (resid 76 and name cb) (resid 76 and name sg) 1.0 -60.0 40.0 2
assign (resid 78 and name n ) (resid 78 and name ca)
      (resid 78 and name cb) (resid 78 and name sg) 1.0 -60.0 40.0 2
assign (resid 87 and name n ) (resid 87 and name ca)
      (resid 87 and name cb) (resid 87 and name cg) 1.0 -60.0 40.0 2
assign (resid 92 and name n ) (resid 92 and name ca)
      (resid 92 and name cb) (resid 92 and name sg) 1.0 -60.0 40.0 2

```

**! phi restraints**

```

assign (resid 14 and name c ) (resid 15 and name n )
      (resid 15 and name ca) (resid 15 and name c ) 1.0 60.0 50.0 2 !HN(CO)HB
assign (resid 18 and name c ) (resid 19 and name n )
      (resid 19 and name ca) (resid 19 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.9 Hz
assign (resid 27 and name c ) (resid 28 and name n )
      (resid 28 and name ca) (resid 28 and name c ) 1.0 -120.0 70.0 2 !HNHA 7.6 Hz
assign (resid 29 and name c ) (resid 30 and name n )
      (resid 30 and name ca) (resid 30 and name c ) 1.0 -78.0 50.0 2
assign (resid 31 and name c ) (resid 32 and name n )
      (resid 32 and name ca) (resid 32 and name c ) 1.0 -34.0 50.0 2
assign (resid 32 and name c ) (resid 33 and name n )
      (resid 33 and name ca) (resid 33 and name c ) 1.0 60.0 50.0 2 !HN(CO)HB
assign (resid 33 and name c ) (resid 34 and name n )
      (resid 34 and name ca) (resid 34 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.2 Hz
assign (resid 34 and name c ) (resid 35 and name n )
      (resid 35 and name ca) (resid 35 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.45 Hz
assign (resid 36 and name c ) (resid 37 and name n )
      (resid 37 and name ca) (resid 37 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.6 Hz
assign (resid 38 and name c ) (resid 39 and name n )
      (resid 39 and name ca) (resid 39 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.6 Hz
assign (resid 39 and name c ) (resid 40 and name n )
      (resid 40 and name ca) (resid 40 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.3 Hz

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assign (resid 40 and name c ) (resid 41 and name n )
      (resid 41 and name ca) (resid 41 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.8 Hz
assign (resid 41 and name c ) (resid 42 and name n )
      (resid 42 and name ca) (resid 42 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.5 Hz
assign (resid 49 and name c ) (resid 50 and name n )
      (resid 50 and name ca) (resid 50 and name c ) 1.0 -75.0 50.0 2
assign (resid 50 and name c ) (resid 51 and name n )
      (resid 51 and name ca) (resid 51 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.5 Hz
assign (resid 52 and name c ) (resid 53 and name n )
      (resid 53 and name ca) (resid 53 and name c ) 1.0 60.0 50.0 2 !HN(CO)HB
assign (resid 55 and name c ) (resid 56 and name n )
      (resid 56 and name ca) (resid 56 and name c ) 1.0 -60.0 50.0 2 !HNHA 5.0 Hz
assign (resid 58 and name c ) (resid 59 and name n )
      (resid 59 and name ca) (resid 59 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.8 Hz
assign (resid 59 and name c ) (resid 60 and name n )
      (resid 60 and name ca) (resid 60 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.2 Hz
assign (resid 60 and name c ) (resid 61 and name n )
      (resid 61 and name ca) (resid 61 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.1 Hz
assign (resid 61 and name c ) (resid 62 and name n )
      (resid 62 and name ca) (resid 62 and name c ) 1.0 -150.0 70.0 2 !HNHA 7.9 Hz
assign (resid 62 and name c ) (resid 63 and name n )
      (resid 63 and name ca) (resid 63 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.7 Hz
assign (resid 73 and name c ) (resid 74 and name n )
      (resid 74 and name ca) (resid 74 and name c ) 1.0 -120.0 50.0 2
assign (resid 74 and name c ) (resid 75 and name n )
      (resid 75 and name ca) (resid 75 and name c ) 1.0 -120.0 50.0 2 !HNHA 9.15 Hz
assign (resid 76 and name c ) (resid 77 and name n )
      (resid 77 and name ca) (resid 77 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.2 Hz

```

## ! psi restraints

```

assign (resid 15 and name n ) (resid 15 and name ca)
      (resid 15 and name c ) (resid 16 and name n ) 1.0 50.0 50.0 2
assign (resid 19 and name n ) (resid 19 and name ca)
      (resid 19 and name c ) (resid 20 and name n ) 1.0 120.0 75.0 2
assign (resid 25 and name n ) (resid 25 and name ca)
      (resid 25 and name c ) (resid 26 and name n ) 1.0 120.0 75.0 2
assign (resid 27 and name n ) (resid 27 and name ca)
      (resid 27 and name c ) (resid 28 and name n ) 1.0 120.0 75.0 2
assign (resid 28 and name n ) (resid 28 and name ca)
      (resid 28 and name c ) (resid 29 and name n ) 1.0 120.0 75.0 2
assign (resid 29 and name n ) (resid 29 and name ca)
      (resid 29 and name c ) (resid 30 and name n ) 1.0 120.0 60.0 2
assign (resid 30 and name n ) (resid 30 and name ca)
      (resid 30 and name c ) (resid 31 and name n ) 1.0 125.0 75.0 2
assign (resid 31 and name n ) (resid 31 and name ca)
      (resid 31 and name c ) (resid 32 and name n ) 1.0 120.0 75.0 2
assign (resid 32 and name n ) (resid 32 and name ca)
      (resid 32 and name c ) (resid 33 and name n ) 1.0 174.0 50.0 2
assign (resid 33 and name n ) (resid 33 and name ca)
      (resid 33 and name c ) (resid 34 and name n ) 1.0 60.0 50.0 2
assign (resid 34 and name n ) (resid 34 and name ca)
      (resid 34 and name c ) (resid 35 and name n ) 1.0 159.0 50.0 2
assign (resid 35 and name n ) (resid 35 and name ca)
      (resid 35 and name c ) (resid 36 and name n ) 1.0 156.0 50.0 2
assign (resid 36 and name n ) (resid 36 and name ca)
      (resid 36 and name c ) (resid 37 and name n ) 1.0 120.0 60.0 2
assign (resid 37 and name n ) (resid 37 and name ca)
      (resid 37 and name c ) (resid 38 and name n ) 1.0 120.0 60.0 2
assign (resid 40 and name n ) (resid 40 and name ca)
      (resid 40 and name c ) (resid 41 and name n ) 1.0 120.0 60.0 2
assign (resid 41 and name n ) (resid 41 and name ca)
      (resid 41 and name c ) (resid 42 and name n ) 1.0 120.0 60.0 2
assign (resid 42 and name n ) (resid 42 and name ca)
      (resid 42 and name c ) (resid 43 and name n ) 1.0 120.0 60.0 2
assign (resid 43 and name n ) (resid 43 and name ca)
      (resid 43 and name c ) (resid 44 and name n ) 1.0 120.0 60.0 2
assign (resid 45 and name n ) (resid 45 and name ca)

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      (resid 45 and name c ) (resid 46 and name n ) 1.0  -60.0  50.0  2 !HNHB
assign (resid 51 and name n ) (resid 51 and name ca)
      (resid 51 and name c ) (resid 52 and name n ) 1.0  120.0  75.0  2
assign (resid 53 and name n ) (resid 53 and name ca)
      (resid 53 and name c ) (resid 54 and name n ) 1.0   50.0  50.0  2
assign (resid 58 and name n ) (resid 58 and name ca)
      (resid 58 and name c ) (resid 59 and name n ) 1.0  -60.0  50.0  2 !HNHB
assign (resid 59 and name n ) (resid 59 and name ca)
      (resid 59 and name c ) (resid 60 and name n ) 1.0   33.0  50.0  2
assign (resid 60 and name n ) (resid 60 and name ca)
      (resid 60 and name c ) (resid 61 and name n ) 1.0  162.0  50.0  2
assign (resid 61 and name n ) (resid 61 and name ca)
      (resid 61 and name c ) (resid 62 and name n ) 1.0  120.0  60.0  2
assign (resid 62 and name n ) (resid 62 and name ca)
      (resid 62 and name c ) (resid 63 and name n ) 1.0  120.0  60.0  2
assign (resid 63 and name n ) (resid 63 and name ca)
      (resid 63 and name c ) (resid 64 and name n ) 1.0  165.0  50.0  2
assign (resid 74 and name n ) (resid 74 and name ca)
      (resid 74 and name c ) (resid 75 and name n ) 1.0  120.0  60.0  2
assign (resid 75 and name n ) (resid 75 and name ca)
      (resid 75 and name c ) (resid 76 and name n ) 1.0  120.0  60.0  2
assign (resid 76 and name n ) (resid 76 and name ca)
      (resid 76 and name c ) (resid 77 and name n ) 1.0  120.0  60.0  2
assign (resid 78 and name n ) (resid 78 and name ca)
      (resid 78 and name c ) (resid 79 and name n ) 1.0  120.0  60.0  2
assign (resid 83 and name n ) (resid 83 and name ca)
      (resid 83 and name c ) (resid 84 and name n ) 1.0  120.0  60.0  2
assign (resid 92 and name n ) (resid 92 and name ca)
      (resid 92 and name c ) (resid 93 and name n ) 1.0  120.0  75.0  2

```

**! chi-2 restraints**

```

assign (resid 2 and name ca) (resid 2 and name cb)
      (resid 2 and name cg1) (resid 2 and name cd1) 1.0  -60.0  40.0  2 !LRCH
assign (resid 31 and name ca) (resid 31 and name cb)
      (resid 31 and name cg) (resid 31 and name cd1) 1.0  180.0  40.0  2 !LRCH
assign (resid 32 and name ca) (resid 32 and name cb)
      (resid 32 and name cg) (resid 32 and name cd1) 1.0  180.0  40.0  2 !LRCH
assign (resid 74 and name ca) (resid 74 and name cb)
      (resid 74 and name cg1) (resid 74 and name cd1) 1.0  -60.0  40.0  2 !LRCH
assign (resid 90 and name ca) (resid 90 and name cb)
      (resid 90 and name cg1) (resid 90 and name cd1) 1.0  180.0  40.0  2 !LRCH

```

**Example 3: Identification of blocking antibodies using a competitive binding assay and immobilised wild type GST-MSP-1<sub>19</sub>.**

In previous studies antibodies that blocked the action of the neutralising antibodies 12.8 and 12.10 had been defined either directly in the MSP-1<sub>42</sub> processing assay (Blackman *et al.*, 1994) in a coupled erythrocyte invasion-MSP-1<sub>42</sub> processing assay (Guevara *et al.*, 1997) or in a competitive radioimmunoassay with merozoite protein as the antigen (Guevara *et al.*, 1997). These studies have been extended using recombinant MSP-1 and BIAcore analysis.

A recombinant fusion protein comprising wild type MSP-1<sub>19</sub> fused to GST was coupled to the sensor chip and competitor antibody was first allowed to bind to the antigen. Then a solution of either mAb 12.8 or 12.10 was passed over the chip and the amount of binding of this second antibody was quantified. If the first antibody interferes with the binding of the second antibody then this is reflected in a reduction in the amount of second antibody bound.

Methods

The wild type GST-MSP-1<sub>19</sub> was coupled to a CM5 sensor chip. The binding assays were performed with a constant flow rate of 5  $\mu\text{l min}^{-1}$  at 25°C. For binding, purified mAbs 1E1, 8A12 and 2F10 at 100  $\mu\text{g ml}^{-1}$  in HBS-EP buffer (10mM HEPES pH7.4 containing 150mM NaCl, 3mM EDTA and 0.005%v/v polysorbate 20); mAbs 1E8, 9C8, 12D11, 111.2 and 111.4 in cell culture medium supernatant; mAbs 2.2, 7.5 and 89.1 at 1:10 dilution of ascitic fluid in HBS-EP buffer; and mouse  $\alpha$ -GST antibody at 1:10 dilution serum in HBS-EP buffer were allowed to interact with immobilised wild type GST-MSP-1<sub>19</sub> for 10 min. After allowing 5 min for dissociation of low affinity interactions, either mAb 12.8 or 12.10 at 100  $\mu\text{g ml}^{-1}$  in HBS-EP buffer was added and allowed to bind for 10 min. After washing the chip for 5 min the binding of 12.8 or 12.10 was measured. The chip was regenerated by washing off bound antibody with 10 mM glycine-HCl, pH 2.4, or when required with 100 mM glycine-HCl, pH 1.8, for 3 min.

### Results

The results are shown in Figure 12. All the competitor antibodies bind to the GST-MSP-1<sub>19</sub> antigen, with the exception of mAb89.1 which is a negative control. As expected, mAbs 12.8 and 12.10 competed with each other (Guevara *et al.*, 1997). The other antibodies which do not inhibit processing could to greater or lesser interfere with the binding of 12.8 and 12.10. As expected from previous studies mAbs 1E1, and 7.5 blocked both 12.8 and 12.10, whereas 2.2 and 111.4 blocked 12.8. Another particularly effective blocking antibody identified in this study was mAb9C8.

### **Example 4: Immunization of small animals with modified GST-MSP-1<sub>19</sub> and analysis of the antibodies induced**

To determine whether or not the modified proteins were immunogenic, recombinant GST-MSP-1<sub>19</sub> fusion proteins were used to raise antibodies by immunisation.

### Methods

Two modified proteins containing either 3 [27+31+43] or 4 [15+27+31+43] amino acid substitutions, respectively, were used to immunise rabbits and mice. The rabbits were immunised subcutaneously with MSP-1<sub>19</sub> protein in Freund's complete adjuvant and then boosted on three occasions with 200 µg of the protein in Freund's incomplete adjuvant 21, 42 and 63 days later, and serum samples were collected.

The presence and level of antibodies binding to the native MSP-1 protein in the parasite was assessed by indirect immunofluorescence using acetone fixed smears of parasite-infected erythrocytes. The sera were diluted serially in phosphate buffered saline (PBS) and incubated on the slide for 30 min at room temperature. After washing, the slides were incubated with FITC conjugated goat anti-rabbit or anti-mouse IgG, washed, and then examined by fluorescence microscopy.

The sera were also analysed in an MSP-1 secondary processing assay. Analysis and quantitation of secondary processing of MSP-1 in merozoite preparations was by a

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modification of an assay described previously (Blackman *et al.*, 1994). Washed *P. falciparum* 3D7 merozoites were resuspended in ice-cold 50 mM Tris-HCl pH 7.5 containing 10 mM CaCl<sub>2</sub> and 2 mM MgCl<sub>2</sub> (reaction buffer). Aliquots of about  $1 \times 10^9$  merozoites were dispensed into 1.5 ml centrifuge tubes on ice, and the parasites pelleted in a microfuge at 13,000 x g for 2 minutes at 4°C. The supernatant was removed, and individual merozoite pellets were then resuspended on ice in 25 µl of reaction buffer further supplemented with protease inhibitor or antibodies as appropriate. Merozoites were maintained on ice for 20 min to allow antibody binding, then transferred to a 37°C water bath for one hour to allow processing to proceed. Assays always included the following controls: a "positive processing" control sample of merozoites resuspended in reaction buffer only; a "negative processing" control sample of merozoites resuspended in reaction buffer plus 1mM PMSF; and a zero time (0h) control, in which processing was stopped before the 37°C incubation step. The processing was assayed using the western blot-based method and by a modified processing assay. Supernatants from the assays were obtained after centrifugation for 30 min at 4°C, 13,000 x g to remove the insoluble material. The amount of MSP-1<sub>33</sub> in the supernatants was measured using an ELISA method. Fifty microlitres of diluted sample supernatants were added to the wells of an ELISA plate (NUNC F96 Cert. Maxisorp) that had been coated with 100 µl/well of 4 µg ml<sup>-1</sup> human mAb X509 in PBS. Plates were incubated for 4 hours at 37°C and then washed 3 times with 0.01% PBS-Tween (PBS-T). Bound MSP-1<sub>33</sub> protein was detected by addition of 100 µl of 1:4000 dilution of mouse mAb G13 for 1 hour at 37°C, followed by washing and the addition of 100 µl of 1:1000 dilution of sheep anti-mouse IgG (H+L) HRP-conjugated antibody. After incubation for 1 h at 37°C, the plates were washed again and HRP was detected by the addition of 100 µl of freshly prepared substrate solution (400mg l<sup>-1</sup> o-phenylenediamine dihydrochloride in 0.05 M phosphate buffer, 0.024 M citric acid and 0.012% H<sub>2</sub>O<sub>2</sub>) at room temperature for 20 min. The reaction was stopped by adding 10 µl of 1 M sulphuric acid and the absorbance of each sample was measured at 492 nm.

## Results

The results are shown in Figure 13. The two modified proteins produced antibodies that reacted with MSP-1 in the parasite-infected erythrocyte, with a serum titre of 1:10,000,

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which was an identical titre to that of a serum produced in the same way by immunisation with a recombinant protein containing the wildtype MSP-1 sequence. This indicates that the modified proteins can produce antibodies that react with the native protein. The antibodies induced by immunization were able to partially inhibit processing at the concentration used in a preliminary experiment, whereas in the control serum no antibodies that inhibited processing were present.

**Example 5: Design and Synthesis of a *Plasmodium Falciparum* Merozoite Surface Protein-1 Gene Fragment Optimized for *Pichia Pastoris* Heterologous Expression**

The coding sequence of the *Plasmodium falciparum* merozoite surface protein-1 (MSP1) 41.1 kDa processed fragment (MSP-142) has been redesigned for optimal heterologous expression in the yeast *Pichia pastoris*. The optimized DNA sequence was synthesized by PCR gene assembly, in the form of two fragments, MSP-133 and MSP-119. *P. pastoris* was transformed with an expression vector containing the optimized MSP-119 construct. Recombinant strains were shown to express high levels of non-glycosylated, properly folded MSP-119 protein.

Proteins encoded by the AT-rich genome of the human malaria parasite *Plasmodium falciparum* are generally poorly expressed in heterologous systems (Withers-Martinez et al., 1999). The methylotrophic yeast *Pichia (Komagataella) pastoris* is an appropriate system for expression of disulphide-bridged proteins such as the C-terminal fragment of the *P. falciparum* merozoite surface protein-1 (MSP1) (White et al., 1994; Morgan et al., 1999). In the *P. pastoris* expression system, it is important to avoid premature transcription termination due to AT-rich stretches (Romanos et al., 1991). Codon preferences for highly expressed genes in *P. pastoris* have been identified (Sreekrishna et al., EP 0 586 892 A1). Therefore, a synthetic MSP-142 gene fragment with codon usage optimized for *P. pastoris* expression was designed, using novel computer software (Withers-Martinez et al., 1999). It has previously been shown that the MSP-119 fragment is partially glycosylated when expressed in *P. pastoris*, and the carbohydrate must be enzymatically removed during purification (Morgan et al., 1999). Therefore, two specific point mutations were introduced into the synthetic MSP-142 protein sequence in order to

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prevent N-linked glycosylation at NxS/T sites (one potential site within the MSP-133 sequence, and a known site within the MSP-119 sequence at Asn 1).

The optimized MSP-142 sequence was synthesized by gene assembly polymerase chain reaction (Stemmer et al., 1995. Withers-Martinez et al., 1999), in the form of separate MSP-133 and MSP-119 fragments. The optimized MSP-119 fragment was subcloned into a novel modified *Pichia* expression vector, transformed into the *P. pastoris* host strain SMD1168, and several independent transformants were isolated. There transformants were shown to efficiently express non-glycosylated, properly folded MSP-119. Strong expression of the optimized gene was observed in low copy number transformants. A multiple copy transformant with intermediate level G418 resistance gave expression of purified MSP-119 at a level equal to the high-expressing strain previously described (Morgan et al., 1999), which contains the original *P. falciparum* DNA. Thus, it should be possible to obtain even higher yields from high level G418-resistant transformants of the synthetic optimized gene.

#### Methods: Gene Assembly

The *P. falciparum* MSP-142 (41.1 kDa) fragment protein sequence SWISS-PROT accession number P04933: positions 1264-1621) was first altered to eliminate N-linked glycosylation signals by 2 amino acid substitutions. The sequences NYT (in the N-terminal portion; position 1445) and NIS (at the beginning of the C-terminal fragment; position 1526) were changed to QYT and NIA respectively. The protein sequence was then reverse-translated with DNA-STAR using the *S. cerevisiae* codon preferences. This sequence was used as input for the CODOP program (Withers-Martinez et al., 1999). Ten random sequences were generated with this program, using a codon weighting table (Figure 14) derived from codon usage in highly expressed *P. pastoris* genes (Sreekrishna et al., EP 0 586 892 A1). Thus, the codon table should reflect usage in highly expressed genes, rather than average usage. The random sequence that contained the minimum number of unfavourable codons (6) was selected, and these codons were changed manually to more preferred alternatives. The sequence was then analysed with DNA-STAR to check for AT-rich sequences that may cause transcription termination, and for



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direct and inverted repeats. A set of 50 overlapping oligonucleotides coding for the final sequence was then generated. This consisted of 49 oligonucleotides of length 42 nt, and one of length 48 nt. Each oligonucleotide had a 21 bp overlap with its neighbours, with no gaps. Estimated  $T_m$ s were in the range of 60°C to 77°C. Oligonucleotides were synthesised by Oswel (Southampton, UK) at 40 nmol scale, and supplied in deionised water without purification. Outside primers of various lengths for the amplification step were also synthesised, to give a  $T_m$  of 62°C to 64°C, and contained a 5'-terminal phosphate group for ligation following the amplification step. The reverse primers also included a translation termination codon (UAA in the complementary strand). All oligonucleotides were diluted to 10  $\mu$ M in ddH<sub>2</sub>O before use.

The PCR-mediated gene assembly and amplification were carried out as described (Stemmer *et al.*, 1995; Withers-Martinez *et al.*, 1999), using a Biometra cyclor, in thin-walled 200  $\mu$ L tubes, under the following conditions.

Gene assembly reactions (Reaction 1):

50  $\mu$ L volume

2 units Vent polymerase (New England Biolabs)

0.4 mM dNTPs

1 x Vent polymerase buffer

Oligonucleotide mix containing each oligonucleotide at 200 nM

Cycles:

32 cycles (2 h 33 m)

denaturation 94°C 30 s

annealing 52°C 30 s

extension 72°C 3 m

Three fragments of the MSP-142 (41.1 kDa) region were synthesised separately with different outside primers and subsets of the 50 oligonucleotide set:

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N-terminal fragment (bp 1-423) 21 oligonucleotides

middle fragment (bp 337-786) 22 oligonucleotides

C-terminal fragment (bp 787-1074) 14 oligonucleotides

- 5 The C-terminal fragment produces a 10.6 kDa fragment (MSP-119). The N-terminal and middle fragments, which overlap between positions 337 and 423, were subsequently spliced together at the BglII site (371-376) to give a 786 bp fragment that encodes the 30.5 kDa MSP-133 protein.

10 Amplification reactions (Reaction 2):

100 µL volume

10 µL aliquot of the gene assembly reaction

4 units Vent polymerase

15 0.4 mM dNTPs

1 x Vent polymerase buffer

1 µM outside primers

Cycles:

20 32 cycles (2 h 55 m)

denaturation 94°C 45 s

annealing 52°C 45 s

extension 72°C 3 m

final extension 72°C 5 m

25

The PCR products were then purified by filtration with Centricon-100 units (Amicon), and cloned directly into the vectors by blunt-end ligation overnight at 16°C with T4 DNA ligase.

- 30 The synthetic MSP-119 gene was cloned directly into a *P. pastoris* expression vector. The modified pPIC9KHXa vector, containing a His<sub>6</sub> tag and factor Xa cleavage site (see Figure 15) inserted in the pPIC9K SnaBI site, had been digested with PmlI and treated

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with calf alkaline phosphatase. The HXa vector had been previously created by insertion of a 36 bp synthetic oligonucleotide, containing the His<sub>6</sub> tag, factor Xa cleavage site, and PmlI restriction site into the SnaBI site of the pPIC9K vector.

5 The N-terminal and middle fragment PCR products were cloned into the SmaI site of the dephosphorylated pUC118 vector. Plasmid clones containing inserts were sequenced. Clones with the correct synthetic sequence were then digested and the two fragments were gel-purified. The N-terminal fragment clones were digested with EcoRI and BglII, and the middle fragment clones were digested with HindIII and BglII. The recombinant fragments  
10 were purified on an agarose gel, and eluted with a QIAGEN extraction kit. The purified N-terminal and middle fragments were then spliced together by ligation into a pUC118 vector that had been digested with HindIII and EcoRI and treated with calf alkaline phosphatase. This created the complete synthetic MSP-133 coding sequence. The N-terminal and middle fragment PCR products were cloned into the SmaI site of the  
15 dephosphorylated pUC118 vector. Plasmid clones containing inserts were sequenced. Clones with the correct synthetic sequence were then digested and the two fragments were gel-purified. The N-terminal fragment clones were digested with EcoRI and BglII, and the middle fragment clones were digested with HindIII and BglII. The recombinant fragments were purified on an agarose gel, and eluted with a QIAGEN extraction kit. The purified  
20 N-terminal and middle fragments were then spliced together by ligation into a pUC118 vector that had been digested with HindIII and EcoRI and treated with calf alkaline phosphatase. This created the complete synthetic MSP-133 coding sequence.

#### Methods: Expression and Purification

25

The methylotrophic yeast *Pichia* (*Komagataella*) *pastoris* strain SMD1168 was transformed by electroporation as described previously (Morgan et al., 1999). In addition, some G418-resistant clones were isolated using Hybond-N+ membranes (Fairlie et al., 1999).

30

Expression screening of transformants was performed by growing 10 ml cultures in buffered minimal glucose medium. Cells were harvested and resuspended in 10 ml

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buffered minimal methanol medium at 1.0 OD<sub>600</sub> and grown overnight to a final OD<sub>600</sub> of 2.5 to 3.0. Cells were removed by centrifugation, and 1.2 ml of the supernatant medium was precipitated 30 min on ice with 15 % trichloroacetic acid. The samples were centrifuged for 30 min at 14000 rpm at 4 °C in a microfuge, and the protein pellets were washed twice with cold acetone. Samples were resuspended in 12 µl ddH<sub>2</sub>O, and 5 µl was electrophoresed, after reduction with DTT, on NOVEX pre-poured acrylamide gels according to manufacturer's instructions. NOVEX 4-12 % acrylamide gradient, or 10 % acrylamide, Bis/Tris gels in MES buffer were used. Protein gels were stained with Coomassie colloidal Brilliant Blue stain (Sigma).

Homogeneously purified MSP-119 was obtained as described previously (Morgan et al., 1999), except that enzymatic deglycosylation was omitted for the synthetic gene products.

#### Methods: NMR

One-dimensional <sup>1</sup>H- and 2-dimensional {<sup>1</sup>H/<sup>15</sup>N}-HSQC spectra were acquired as described previously (Morgan et al., 1999), at 25 °C, at sample concentrations of 1.1-2.5 mM.

#### Results

The sequences of the synthetic DNA fragments, and the resulting predicted protein products, are shown in Figure 15. A summary of the resulting improvements to the sequence is given in Table 3.

		Total codons	<i>P. pastoris</i> preferred codons	Unfavourable codons	% AT content
<i>P. falciparum</i> MSP1	358	140	28	74	
41.1 kDa fragment					
Synthetic 41.1 kDa	358	276	0	58	
fragment					

Table 3. Codon usage

PCR-gene assembly reactions for the MSP-133 (two sections) and MSP-119 synthetic fragments are shown on agarose gels in Figure 16. This demonstrated that a single, correct size major product was observed in each case. The PCR products were subcloned, screened, and sequenced as described in the Methods section.

*P. pastoris* was transformed with the synthetic MSP-119 construct in the modified pPIC9K expression vector (pPIC9K-HXa; Figure 15). Expression of the synthetic MSP-119 product in three independent transformants is shown on a protein gel in Figure 17. The protein samples were prepared by trichloroacetic acid precipitation from culture supernatants as described in the Methods section. This demonstrated that a single, major product was present in each sample, corresponding to the expected migration of the synthetic MSP-119 protein. This migrated slightly more slowly than the control sample, which as described previously (Morgan et al., 1999) has a shorter N-terminal tag sequence. There was no trace of heterogeneous, slowly migrating recombinant protein that would result from glycosylation. Therefore, non-glycosylated, synthetic MSP-119 is efficiently expressed by the transformed yeast. The yield (measured by UV absorbance) of purified MSP-119 was 16 mg/L for low copy number transformants (resistant to 0.25 mg/ml G418), and increased to 24 mg/L for intermediate G418 resistance (resistant to 1.0 mg/ml G418). This can be compared with yields of 1-2 mg/L for low copy number transformants of *P. pastoris* with the original *Plasmodium falciparum* coding sequence.

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before isolation of a highly G418-resistant strain (Morgan et al., 1999). This indicated that the synthetic MSP-119 construct is advantageous for recombinant protein expression, and that further improvement would result from isolation of higher copy number transformants.

5

One-dimensional proton NMR experiments demonstrated that the synthetic MSP-119 protein spectrum was very similar to the previously studied protein (Morgan et al., 1999), and represented a correctly folded protein (data not shown). This was further confirmed by a 2D- $\{^1\text{H}/^{15}\text{N}\}$ -HSQC spectra (Figure 18), which also shows that the structure of the  
10 synthetic product is identical to the previously studied protein, except for slight differences at the N-terminus which are consistent with the presence of a distinct N-terminal tag sequence, and S3->A mutation at the glycosylation site. Backbone NH proton and  $^{15}\text{N}$  chemical shifts for the original *P. falciparum* sequence product have been  
15 previously presented (Morgan et al., 1999). The similarity between the two spectra, outside of the N-terminal region, is strong evidence that both protein forms are in a structurally similar, correctly folded state.

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**CLAIMS**

1. A non-naturally occurring variant of a C-terminal fragment of a *Plasmodium* merozoite surface protein-1 (MSP-1) wherein said variant has (i) a reduced affinity, compared with a naturally occurring *Plasmodium* MSP-1<sub>19</sub>, for at least one first antibody capable of blocking the binding of a second antibody, which second antibody inhibits the proteolytic cleavage of *Plasmodium* MSP-1<sub>42</sub> and (ii) substantially the same affinity for at least one third antibody compared with said naturally occurring *Plasmodium* MSP-1<sub>19</sub>, which third antibody inhibits the proteolytic cleavage of *Plasmodium* MSP-1<sub>42</sub>.

2. A variant according to claim 1 wherein said *Plasmodium* MSP-1<sub>19</sub> and MSP-1<sub>42</sub> are *Plasmodium falciparum* MSP-1<sub>19</sub> and MSP-1<sub>42</sub>.

3. A variant according to claim 1 or 2 wherein the first antibody is selected from mAbs 1E1, 2.2, 7.5, 9C8 and 111.4.

4. A variant according to claim 1 or 2 wherein the second and/or third antibody is selected from mAbs 12.8, 12.10 and 5B1.

5. A non-naturally occurring variant of a C-terminal fragment of a *Plasmodium* merozoite surface protein-1 (MSP-1) comprising an amino acid modification at any one of amino acid residues 14, 15, 27, 31, 34, 43, 48 and 53 of the *Plasmodium falciparum* MSP-1<sub>19</sub> amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

6. A variant according to claim 5 wherein said modifications are selected from Gln14→Arg, Gln14→Gly, Asn15→Arg, Glu27→Tyr, Leu31→Arg, Tyr34→Ser, Tyr34→Ile, Glu43→Leu, Thr48→Lys and Asn53→Arg and their equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

7. A variant according to claim 6 wherein said substitutions are selected from [Glu27→Tyr, Leu31→Arg and Glu43→Leu], [Glu27→Tyr, Leu31→Arg, Tyr34→Ser and Glu43→Leu], [Asn15→Arg, Glu27→Tyr, Leu31→Arg and Glu43→Leu] and their

equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

8. A variant according to any one of claims 5 to 7 further comprising a mutation at Cys12 and/or Cys28 of the *Plasmodium falciparum* MSP-1<sub>19</sub> amino acid sequence shown as SEQ I.D. No. 1.

9. A variant according to claim 8, in which the mutation is a substitution selected from Cys12→Ile and Cys28→Trp, and Cys12→Ala and Cys28→Phe.

10. A variant according to claim 9, in which the substitutions are selected from [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Glu43→Leu], [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Glu43→Leu, Asn53→Arg], [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Tyr34→Ser, Glu43→Leu, Asn53→Arg] and their equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

11. A method for producing a *Plasmodium* MSP-1 variant for use in preparing a vaccine composition which method comprises modifying one or more amino acid residues of a *Plasmodium* MSP-1 C-terminal fragment such that the resulting derivative has (i) a reduced affinity, compared with a naturally occurring *Plasmodium* MSP-1<sub>19</sub>, for at least one first antibody capable of blocking the binding of a second antibody, which second antibody inhibits the proteolytic cleavage of *Plasmodium* MSP-1<sub>42</sub> and (ii) substantially the same affinity for at least one third antibody compared with said naturally occurring *Plasmodium* MSP-1<sub>19</sub>, which third antibody inhibits the proteolytic cleavage of *Plasmodium* MSP-1<sub>42</sub>.

12. A non-naturally occurring *Plasmodium* MSP-1 variant obtained by the method of claim 11.

13. A polynucleotide encoding a variant according to any one of claims 1 to 10 or 12 operably linked to a regulatory sequence capable of directing the expression of said nucleotide in a host cell.

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14. 14. A polynucleotide according to claim 13 having a sequence optimised for expression in said host cell.
15. A polynucleotide according to claim 13 or 14, in which the host cell is a *Pichia*  
5 *pastoris* cell.
16. A nucleic acid vector comprising a polynucleotide according to claim 13, 14 or 15.
17. A host cell comprising a vector according to claim 16.
- 10 18. A pharmaceutical composition comprising a variant according to any one of claims 1 to 10 or 12, a polynucleotide according to any one of claims 13 to 15 or a vector according to claim 16 together with a pharmaceutically acceptable carrier or diluent.
- 15 19. A composition according to claim 18 further comprising an immunogenic *Plasmodium* polypeptide or fragment or derivative thereof.
- 20 20. A method for producing anti-MSP-1 antibodies which method comprises administering a polypeptide according to any one of claims 1 to 10 or 12, a polynucleotide according to any one of claims 13 to 15 or a vector according to claim 16 to a mammal.
- 25 21. A method for producing polyclonal anti-MSP-1 antibodies which method comprises administering a polypeptide according to any one of claims 1 to 10 or 12, a polynucleotide according to any one of claims 13 to 15 or a vector according to claim 16 to a mammal and extracting the serum from said mammal.
22. An antibody produced by the method of claim 20 or 21.
- 30 23. A method of inducing immunity against malaria induced by *Plasmodium falciparum* which comprises administering to a person in need of such immunity an effective amount of the variant of claim 1, the polynucleotide of claim 13 or the vector of claim 16.

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24. A method of immunizing a mammal, said method comprising administering an effective amount the polypeptide of claim 1, the polynucleotide of claim 13 or the vector of claim 16.

5

25. The method of claim 24, wherein said mammal is immunized against malaria.

26. A method of treating a malaria infection in a human patient which comprises administering to the patient an effective amount of the pharmaceutical composition of claim 18 or 19.

10

27. A variant according to any one of claims 1 to 10 or 12, a polynucleotide according to any one of claims 13 to 15 or a vector according to claim 16 for use in therapy.

15

24. A computer readable medium having stored thereon a model of the MSP-1<sub>19</sub> NMR structure.

25. A nucleic acid encoding a *Plasmodium* MSP-1 polypeptide, in which the nucleic acid is optimised for expression in a heterologous host cell.

20

26. A nucleic acid according to Claim 25, in which the heterologous host is a *Pischia pastoris* cell.

27. A nucleic acid according to Claim 25 or 26, in which the polypeptide is selected from an MSP-1<sub>42</sub> polypeptide comprising a sequence shown in Figures 2C and 2E, an MSP-1<sub>19</sub> polypeptide comprising a sequence shown in Figure 2C, and an MSP-1<sub>33</sub> polypeptide comprising a sequence shown in Figure 2E.

25

28. A nucleic acid according to any of Claims 25 to 27, in which the nucleic acid comprises a sequence selected from the sequences of Figure 2A, Figure 2B and Figure 2D.

30



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29. A nucleic acid vector comprising a nucleic acid according to any of Claims 25 to 28.

5 30. A host cell comprising a vector according to claim 29.

31. A pharmaceutical composition comprising a nucleic acid according to any of Claims 25 to 27 or a vector according to Claim 29 together with a pharmaceutically acceptable carrier or diluent.

10

32. A composition according to Claim 31 further comprising an immunogenic *Plasmodium* polypeptide or fragment or derivative thereof.

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	1	10	20	30	40
<i>P. falciparum</i> domain-1	NISQ-HQ	CVK---	KQCPQNSG	ERHLD---	EREECKC
<i>P. falciparum</i> domain-2	-----	TCNEN-NGG	CDADAKC	TEEDSGS	NGKKITCE
	50	60	70	80	90
	-----	CTKPD	SYPLE	DCIF	CSSSN-----
<i>P. vivax</i> domain-1	TMSSEHT	CIDT---	NVPDNA	CYRYLDGT---	EEWRCLIT
<i>P. vivax</i> domain-2	-----	TKDN-NGG	CAPEAE	CKWTD	S-----
	-----	NKIV	CKCTKEG	SEPL	FE
	-----	GVFC	SSSS	-----	-----
legf human epidermal growth factor	--NSYPG	CPSSYDGY	CLNGGV	CMHIES---	LDSYT
	-----	NCV	ICY	-----	SGDR
EGF consensus	---	C---	C---	C---	C---
a = aromatic (Phe or Tyr)	---	C---	C---	C---	C---
reduced core segment for alignment (14 aa)	xxxx	.....	xxxxxxxxxxxx		

FIG. 1

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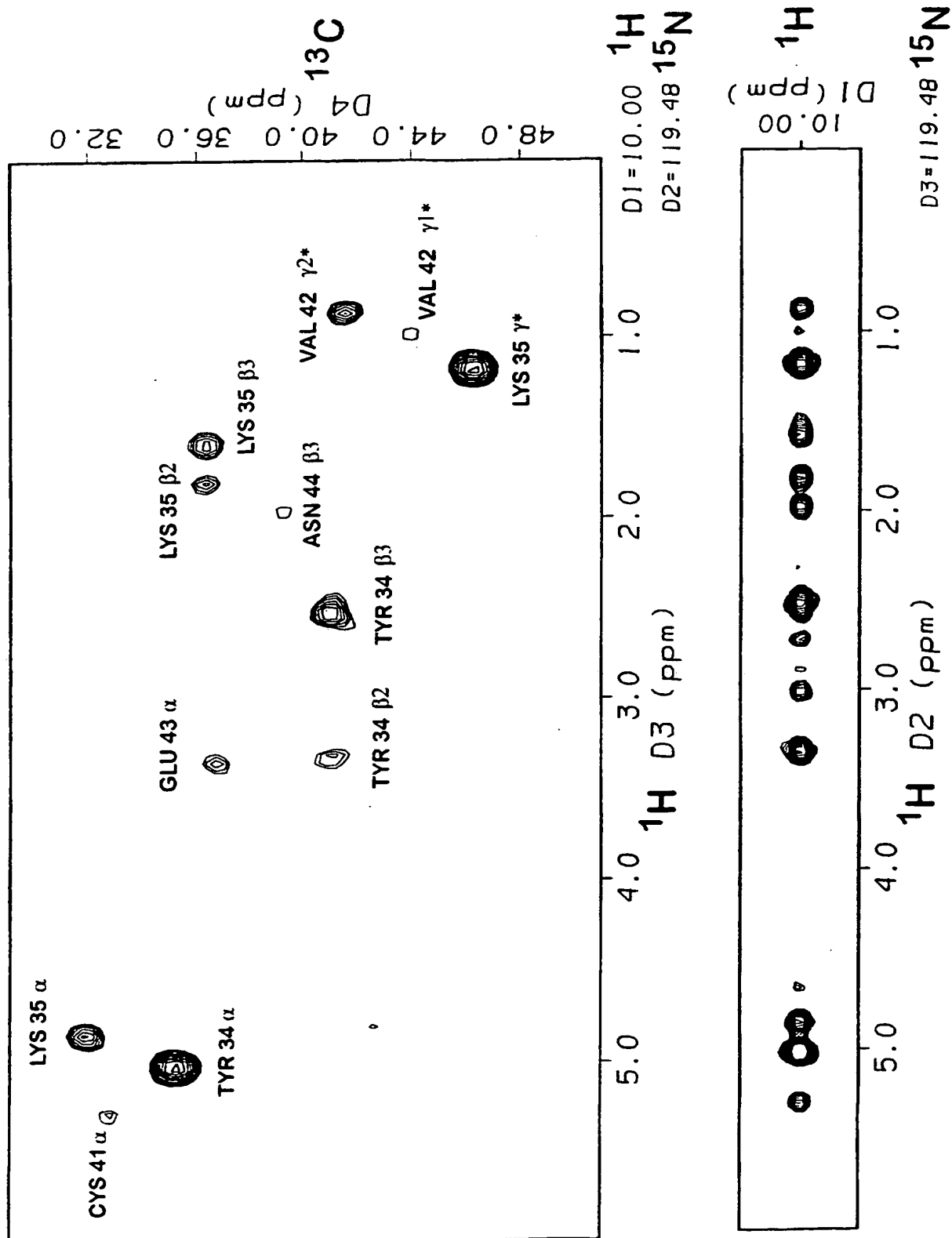


FIG. 2

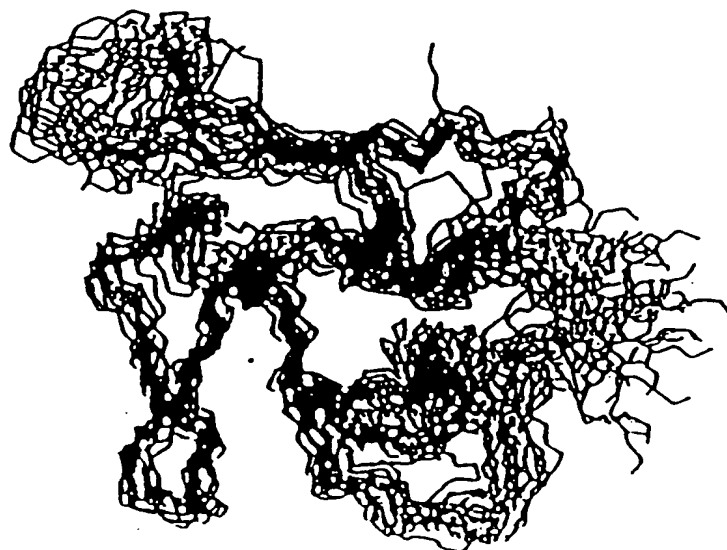
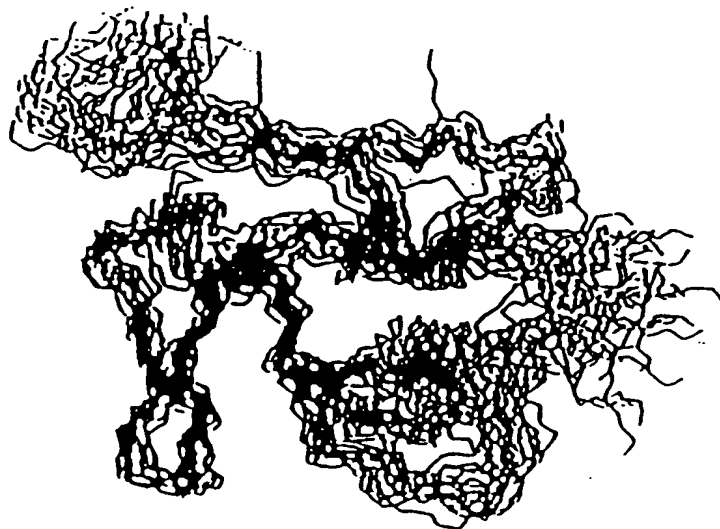


FIG. 3



FIG. 4

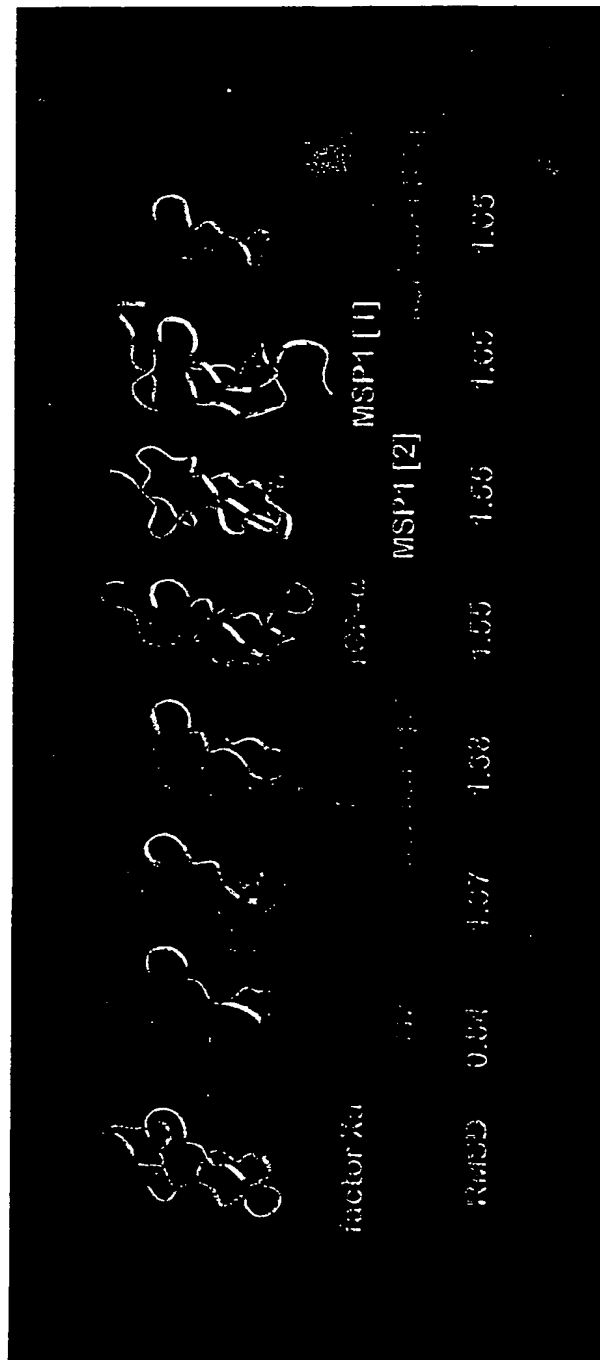


FIG. 5

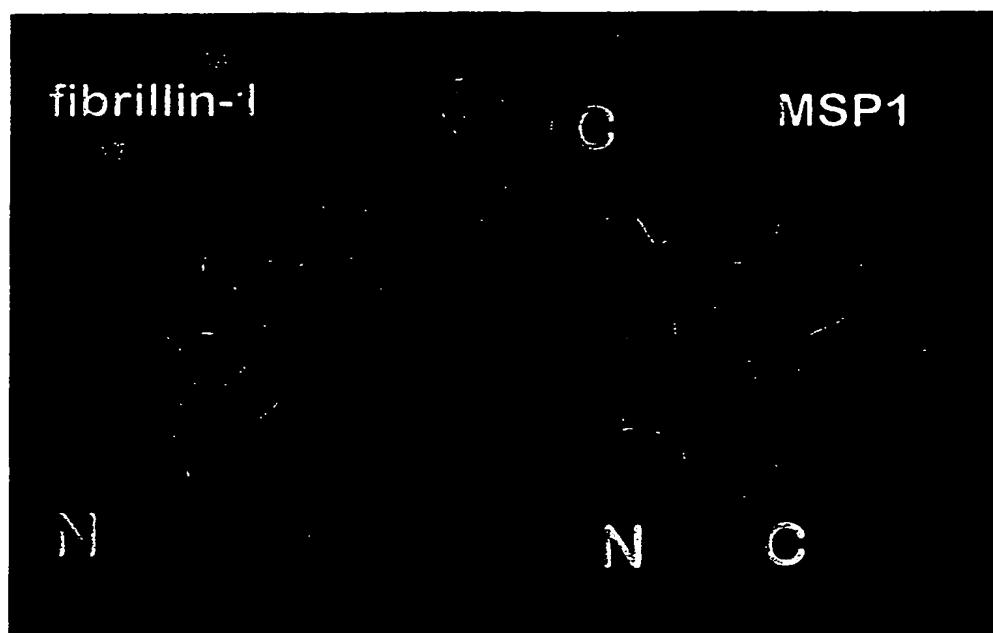


FIG. 6

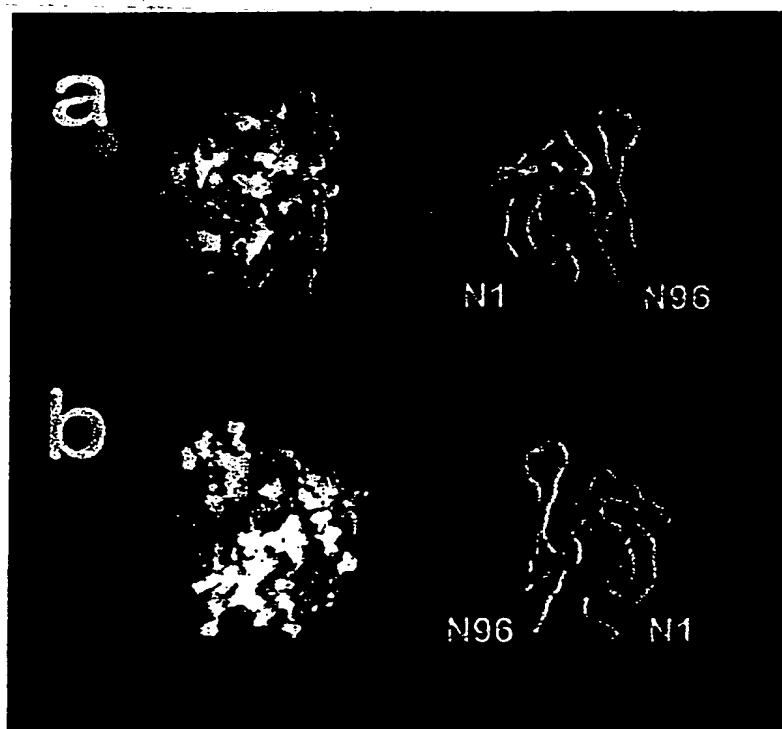


FIG. 7

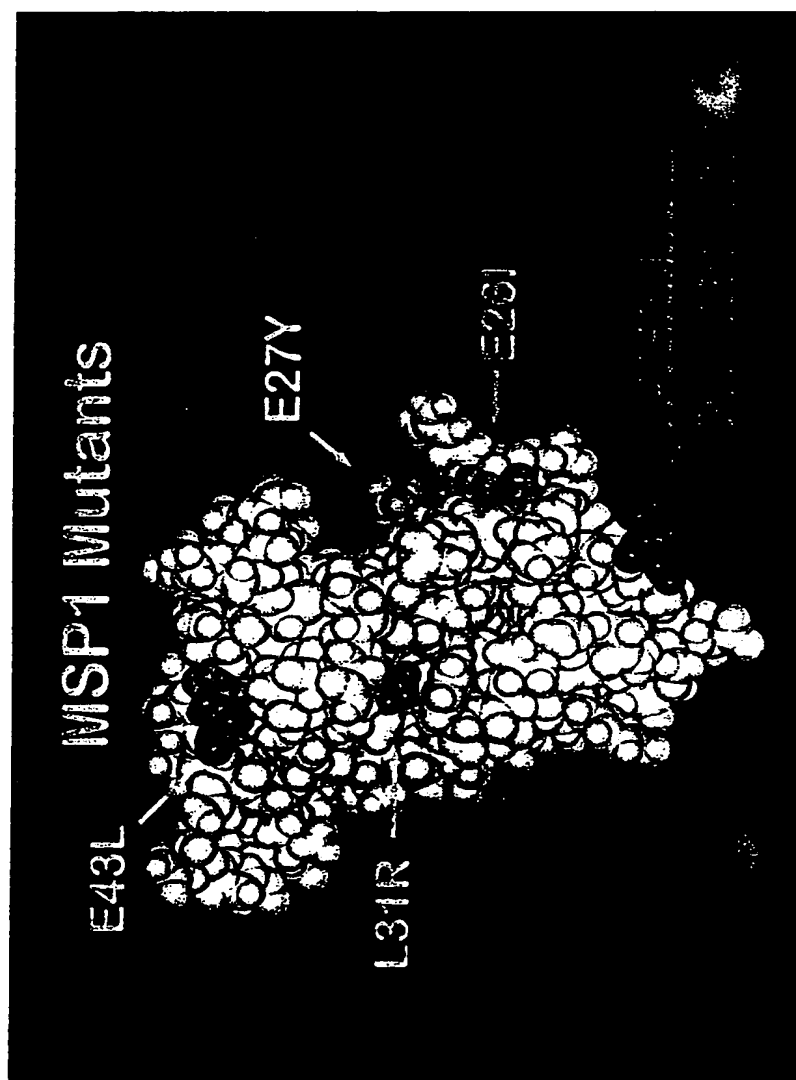


FIG. 8



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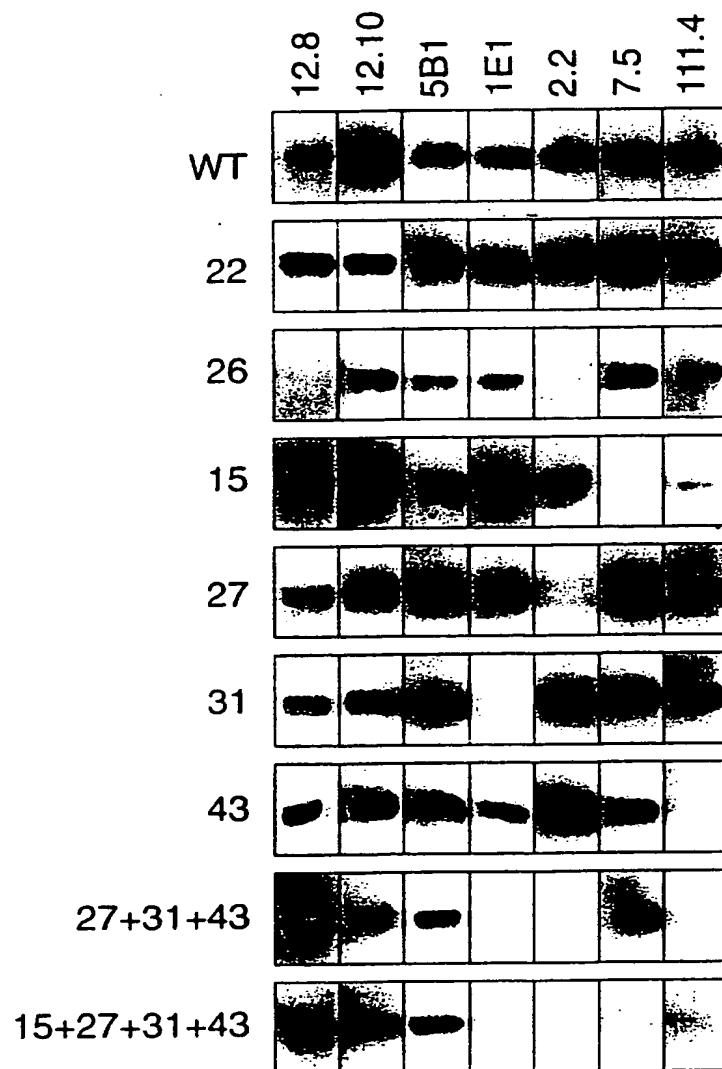


FIG. 9

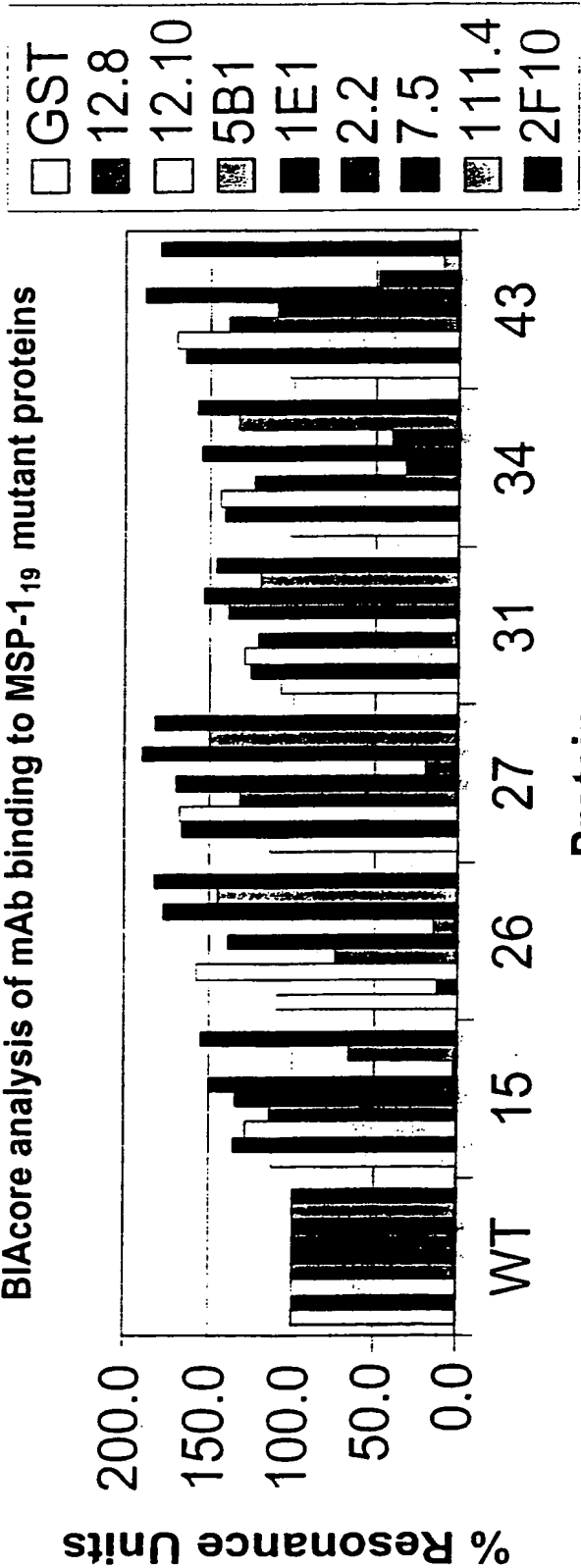
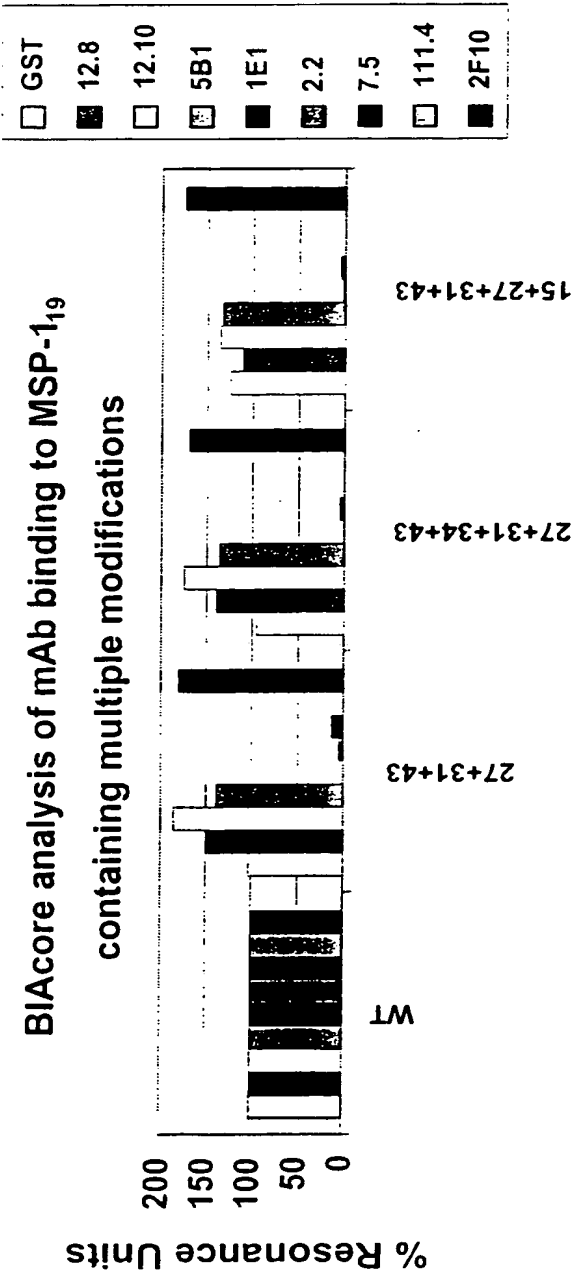


FIG. 10



Protein

FIG. 11

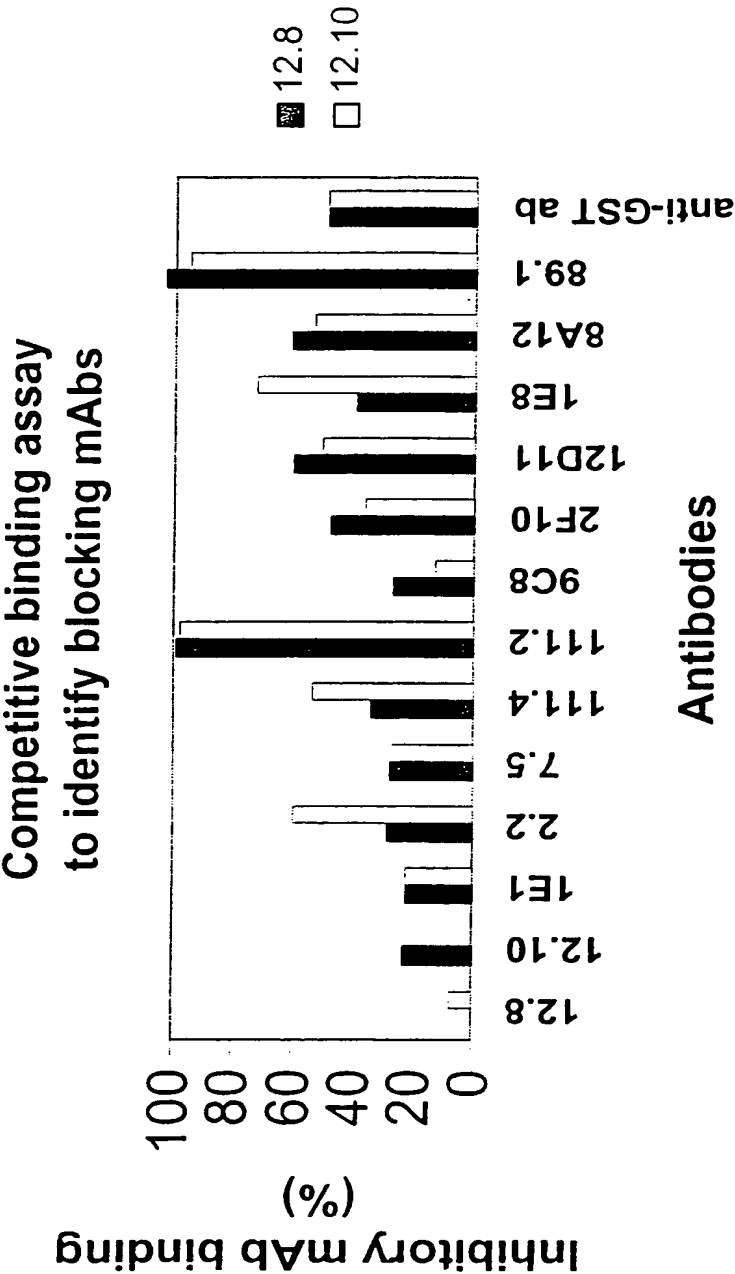


FIG. 12

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### Effect of antibodies in the secondary processing assay

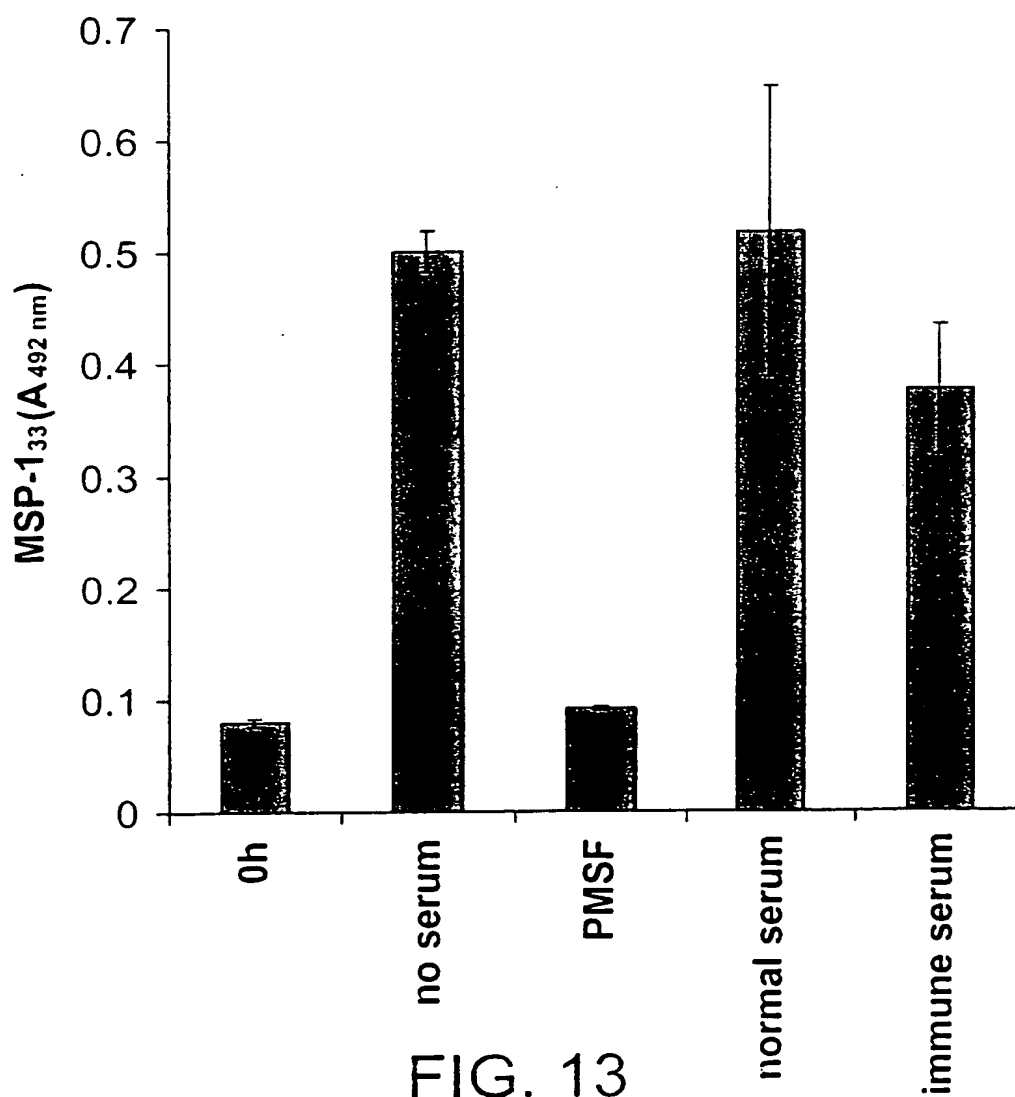


FIG. 13

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= Codon Usage Table for Komagataella (Pichia) pastoris  
 = for use with CODOP program  
 = Fields: [amino acid] [triplet] [frequency per thousand] [number]  
 = codon preferences weights:

=  
 = 30 preferred uniquely  
 = 20 preferred alternative  
 = 10 second best alternative  
 = 15 two equally preferred codons  
 = 1 unclassified  
 = 0 forbidden codons

=  
 = codon selection list

=  
 # Phe TTC  
 # Leu TTG > CTG  
 # Ile ATC = ATT and not ATA  
 # Met ATG  
 # Val GTT > GTC and not GTA  
 # Ser TCT = TCC  
 # Pro CCA > CCT and not CCG  
 # Thr ACT = ACC  
 # Ala GCT > GCC and not GCG  
 # Tyr TAC and not TAT  
 # \* UAA  
 # His CAC > CAT  
 # Gln CAA > CAG  
 # Asn AAC  
 # Lys AAG  
 # Asp GAC > GAT  
 # Glu GAA = GAG  
 # Cys TGT > TGC  
 # Trp TGG  
 # Arg AGA and not CGC . CGA . CGG  
 # Gly GGT > GGA and not GGG

Phe UUU 1.0( 1)	Ser UCU 15.0( 15)	Tyr UAU 0.0( 0)	Cys UGU 20.0( 20)
Phe UUC 30.0( 30)	Ser UCC 15.0( 15)	Tyr UAC 30.0( 30)	Cys UGC 10.0( 10)
Leu UUA 1.0( 1)	Ser UCA 1.0( 1)	Ter UAA 30.0( 30)	Ter UGA 0.0( 0)
Leu UUG 20.0( 20)	Ser UCG 1.0( 1)	Ter UAG 0.0( 0)	Trp UGG 30.0( 30)

Leu CUU 1.0( 1)	Pro CCU 10.0( 10)	His CAU 10.0( 10)	Arg CGU 1.0( 1)
Leu CUC 1.0( 1)	Pro CCC 1.0( 1)	His CAC 20.0( 20)	Arg CGC 0.0( 0)
Leu CUA 1.0( 1)	Pro CCA 20.0( 20)	Gln CAA 20.0( 20)	Arg CGA 0.0( 0)
Leu CUG 10.0( 10)	Pro CCG 0.0( 0)	Gln CAG 10.0( 10)	Arg CGG 0.0( 0)

Ile AUU 15.0( 15)	Thr ACU 15.0( 15)	Asn AAU 1.0( 1)	Ser AGU 1.0( 1)
Ile AUC 15.0( 15)	Thr ACC 15.0( 15)	Asn AAC 30.0( 30)	Ser AGC 1.0( 1)
Ile AUA 0.0( 0)	Thr ACA 1.0( 1)	Lys AAA 1.0( 1)	Arg AGA 30.0( 30)
Met AUG 30.0( 30)	Thr ACG 1.0( 1)	Lys AAG 30.0( 30)	Arg AGG 1.0( 1)

Val GUU 20.0( 20)	Ala GCU 20.0( 20)	Asp GAU 10.0( 10)	Gly GGU 20.0( 20)
Val GUC 10.0( 10)	Ala GCC 10.0( 10)	Asp GAC 20.0( 20)	Gly GGC 1.0( 1)
Val GUA 0.0( 0)	Ala GCA 1.0( 1)	Glu GAA 15.0( 15)	Gly GGA 10.0( 10)
Val GUG 1.0( 1)	Ala GCG 0.0( 0)	Glu GAG 15.0( 15)	Gly GGG 0.0( 0)

FIG. 14

## FIG. 15

- A:**  
 gctgttactccatctgtatcgataaacattctgtcttaagattgagaacgaatacagaggtcttgttacttgaagcctctggc  
 cgggtgtctacagatccctgaagaagcaactggaacaaacgctacgtcttcaacgttaacgltcaaggacattttgaact  
 ccagattcaacaagagagaaacttcaagaacgttctggagctgtgactgtgattccatcaaggatttgacttcttctaac  
 tacgttgttaaggaccatacaagttcctgaacaggaagagagacaagttcttgcctcttacaactacattaaagga  
 tcccatgtatactatcaacttcgttaacagcgtccctgggttactacaagatcttgtctgagaaggtacaagttcgtgact  
 ttgatttccatcaagaagtacatcaatgataagaaggaagagaatgaaaagtacctgccattccctgaataaacattgaaact  
 ttgtacaagactgttaacgataagatcgatctgttcgtcattcatttgaagccaaggtcttgcaatacacattacgaaaa  
 gttaaacgttcgaggtcaagatcaaggaattgaaactacctcaagaccattcaggataagctggctgatttcaagaagaaca  
 acaacttcgttggatcgctgatttgtccactgattacaaccacaacaatctgttgactaagttcctgtctaccggtatg  
 gtttcgagaacttggctaaagactcctgtccactctgttgatggttaactgcagggtgattgaaacattgcccaca  
 ccaatgcyttaagaagcaatgtccacaaaactccggatgtttcagacatcttgacgagagaagaatgtaagtctctgt  
 tgaactacaagcaggaagtataaagtgtgtgagaacccaaacctacctgtaacgagaacaacggtggtgcgacgt  
 gacgttaagtcaccgaagaagactctggttctaacggaagaagattacttgcgaatgtaactaagccagactcttacc  
 tttgttcgatggaattctctgttcttctcttaactaa
- B:**  
 TACCACCATCATCATCATTGAGGTAGACACaacaattgcccaacacccaatgcgttaagaagcaatgtccacaaaaactccgg  
 Atgtttcagacatctggacgagagagaagaatgtaagtgtctgttgaactacaagcaggaagtgataaagtgtgttgagaacccaa  
 Aacctaactgttaacgagaaacacggtggatgcgacgtgagctaaagttaacccaagaagactctggttcttaacggaagaagactt  
 aactgggaatgtactaaagacagactcttaccctttgttcgatggaattcttcttcttcttctc  
 taactaaatGGTA
- C:**  
 YHHHHHHHIEGRHNIAQHQCCKKQCPQNSGCFRHLDEREECKLLNYKQEGDKCVENPNFTCNENNGGCDAD  
 AKTEEDSGSNGKKITCECTKPDSPYPLDFGIFCSSN.
- D:**  
 gctgttactccatctgttatcgataaacattctgtctaaagattgagaacgaatacagaggtcttgttacttgaagcctctggc  
 cgggtgtctacagatccctgaagaagcaactggaacaaacgctacgtcttcaacgttaacgltcaaggacattttgaact  
 ccagattcaacaagagagaaacttcaagaacgttctggagctgtgactgttccatcaaggatttgacttcttctaac  
 tacgttgttaaggaccatacaagttcctgaacaggaagagagacaagttcttgcctcttacaactacattaaagga  
 tcccatgtatactatcaacttcgttaacgacgtccctgggttactacaagatcttgtctgagaaggtacaagttcgtgact  
 ttgatttccatcaagaaggtacatcaatgataagaaggaagagaatgaaaagtacctgccattccctgaataaacattgaaact  
 ttgtacaagactgttaacgatcaagatcgatctgttcgtcattcatttggaaagccaaggtcttgcataacacttaacgaaa  
 gtctaaagtcagaggtcaagatcaaggaattgaactacctcaagaccattcaggaataagctggctgatttcaagaagaaca  
 acaacttgggttggtatcgctgatttgcactgattacaacacacaacattctgtgactaagttcctgtctacacgggtatg  
 gttttctaaagaaacttggctaaagactgtcctgtccaaacctgttgatggtaacttgcagggtatgttg
- E:**  
 AVTPSVIDNILSKIENEYEVLYLKPLAGVYRSLKKQLENNVMTFNVNVKDIILNSRFNFKRENFKNVLES  
 PYKDLTSSNYVVKDPYKFLNKEKRDKFLSSNYIKDSIDTIDINFANDVLGYKILSEKYSKDLDSIKKYIN  
 DKQGENEKYLPFLNNIETLYKTVNDKIDLFVIHLEAKVLQTYEKSNEVEVKIKELNYLKTIQDKLADFKN  
 NNEVGIADLSTDYNHNNLLTKFLSTGMVFENLAKTVLSNLLDGNLQGL.

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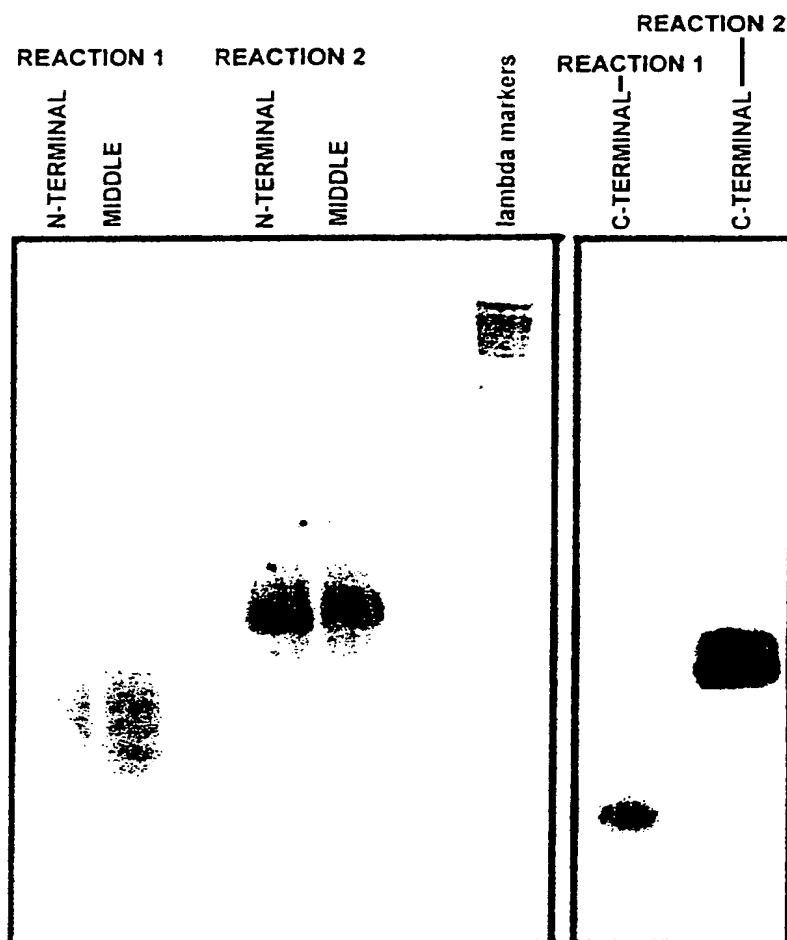
**GENE ASSEMBLY PCR**

FIG. 16



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## EXPRESSION OF SYNTHETIC MSP1-19 GENE IN P. PASTORIS

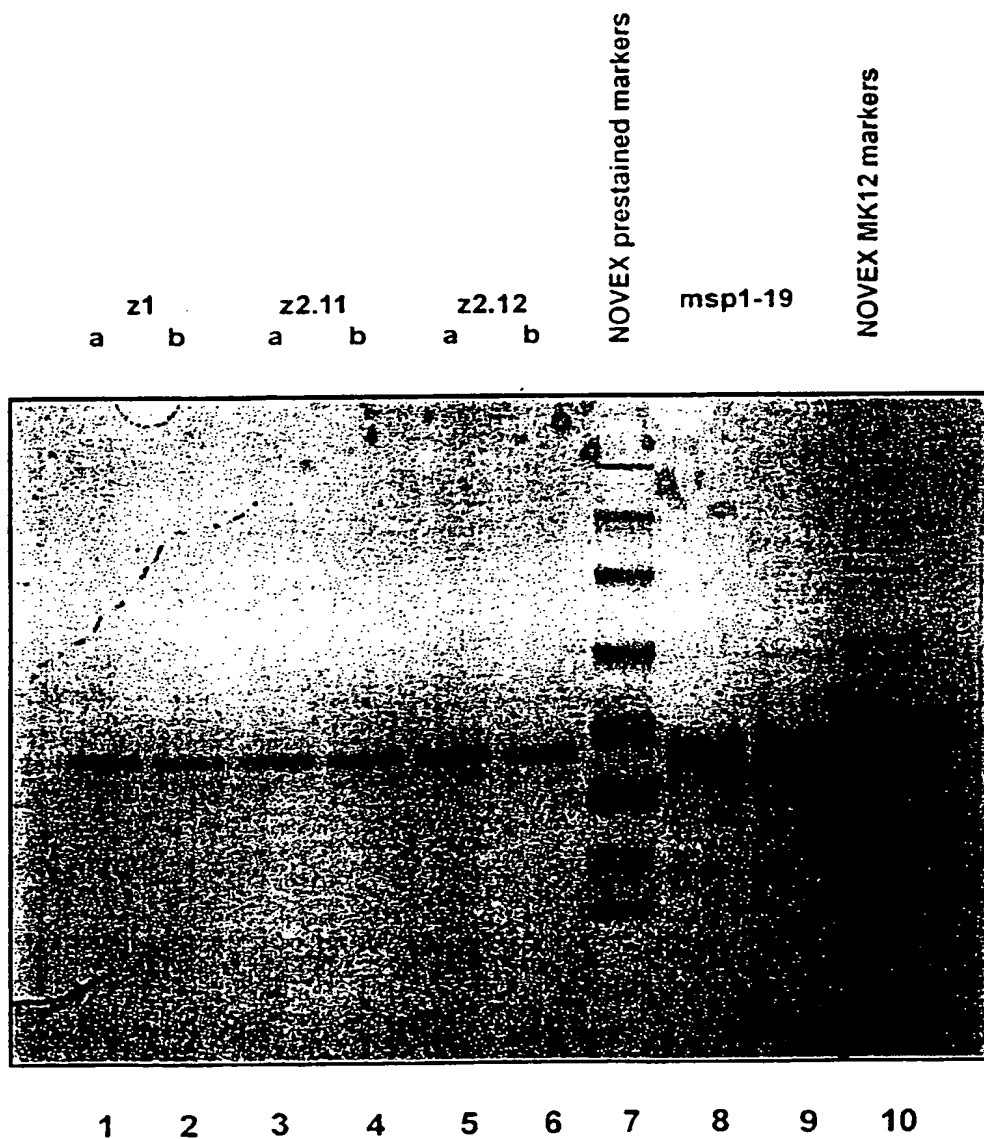


FIG. 17

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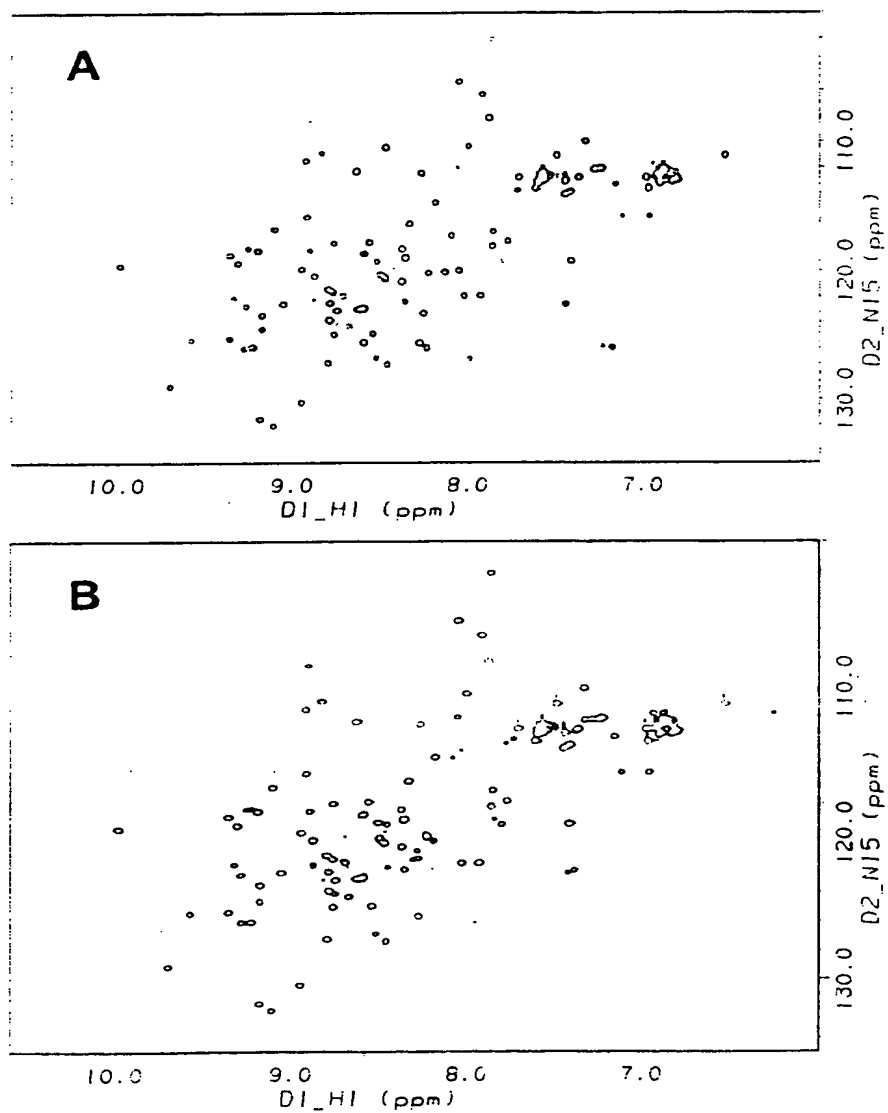


FIG. 18

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International Bureau



(43) International Publication Date  
26 October 2000 (26.10.2000)

PCT

(10) International Publication Number  
**WO 00/63245 A3**

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C12N 15/30, 5/10, A61K 39/00, C07K 16/20, G06F  
17/50, A61K 49/00

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(25) Filing Language: English

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09/311,817 13 May 1999 (13.05.1999) US  
2,271,451 25 May 1999 (25.05.1999) CA

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(74) Agents: **MASCHIO**, Antonio et al.; D. Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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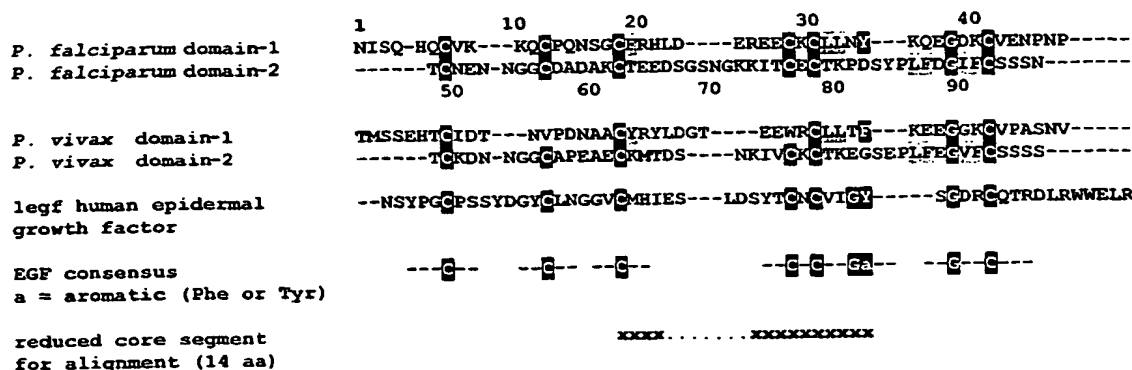
Published:

— With international search report.

(88) Date of publication of the international search report:  
3 May 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: VARIANT OF THE PLASMODIUM MEROZOITE SURFACE PROTEIN (MSP-1) AND VACCINE COMPRISING SAID VARIANT



(57) Abstract: A non-naturally occurring variant of a C-terminal fragment of a *Plasmodium* merozoite surface protein-1 (MSP-1) wherein said variant has (i) a reduced affinity, compared with a naturally occurring *Plasmodium* MSP-1<sub>19</sub>, for at least one first antibody capable of blocking the binding of a second antibody, which second antibody inhibits the proteolytic cleavage of *Plasmodium* MSP-1<sub>42</sub> and (ii) substantially the same affinity for at least one third antibody compared with said naturally occurring *Plasmodium* MSP-1<sub>19</sub>, which third antibody inhibits the proteolytic cleavage of *Plasmodium* MSP-1<sub>42</sub> is provided for use in an anti-malarial vaccine.

WO 00/63245 A3

# INTERNATIONAL SEARCH REPORT

national Application No  
PCT/GB 00/01558

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/445 C12N15/30 C12N5/10 A61K39/00 C07K16/20  
G06F17/50 A61K49/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K A61K G06F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, STRAND, GENSEQ, CHEM ABS Data, MEDLINE, SCISEARCH  
EMBASE, EMBL

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 30158 A (NATO FARIDABANO ;ROTH CHARLES (FR); LONGACRE ANDRE SHIRLEY (FR); P) 21 August 1997 (1997-08-21)  page 14, line 13 -page 15, line 17 page 16, line 5-19 page 33, line 9-23 page 34, line 21-31	1-5, 13, 14, 16-27, 29, 33-36
Y	---	14-17, 30
	--- -/--	

☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

4 December 2000

Date of mailing of the international search report

19/12/2000

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ALCONADA RODRIG., A

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 00/01558

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KASLOW DAVID C ET AL: "Expression and antigenicity of Plasmodium falciparum major merozoite surface protein (MSP1-19) variants secreted from Saccharomyces cerevisiae." MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 63, no. 2, 1994, pages 283-289, XP000603953 ISSN: 0166-6851	1-5, 13-27, 29,33-36
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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 00/01558

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Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PAN WEIQING ET AL: "Vaccine candidate MSP-1 from Plasmodium falciparum: A redesigned 4917 bp polynucleotide enables synthesis and isolation of full-length protein from Escherichia coli and mammalian cells." NUCLEIC ACIDS RESEARCH, vol. 27, no. 4, 15 February 1999 (1999-02-15), pages 1094-1103, XP000953100 ISSN: 0305-1048 the whole document	29,33-36
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A	--- PERERA K L R LAKSHMAN ET AL: "Baculovirus merozoite surface protein 1 C-terminal recombinant antigens are highly protective in a natural primate model for human Plasmodium vivax malaria." INFECTION AND IMMUNITY, vol. 66, no. 4, April 1998 (1998-04), pages 1500-1506, XP002153977 ISSN: 0019-9567 the whole document --- -/--	20,23-26

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 00/01558

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BLACKMAN MICHAEL J ET AL: "Antibodies inhibit the protease-mediated processing of a malaria merozoite surface protein." JOURNAL OF EXPERIMENTAL MEDICINE, vol. 180, no. 1, 1994, pages 389-393, XP000957584 ISSN: 0022-1007 cited in the application figures 2,3 page 391, left-hand column -page 392, left-hand column, paragraph 1</p> <p style="text-align: center;">----</p>	1-4
A	<p>PATINO JOSE A GUEVARA ET AL: "Antibodies that inhibit malaria merozoite surface protein-1 processing and erythrocyte invasion are blocked by naturally acquired human antibodies." JOURNAL OF EXPERIMENTAL MEDICINE, vol. 186, no. 10, 17 November 1997 (1997-11-17), pages 1689-1699, XP000957550 ISSN: 0022-1007 figures 1,2</p> <p style="text-align: center;">----</p>	1-4
P,X	<p>LALITHA P V ET AL: "Plasmodium falciparum: Variations in the C-terminal cysteine-rich region of the merozoite surface protein-1 in field samples among Indian isolates." EXPERIMENTAL PARASITOLOGY, vol. 92, no. 1, May 1999 (1999-05), pages 12-18, XP000957557 ISSN: 0014-4894 page 15, left-hand column, last paragraph -page 15, right-hand column; figure 2</p> <p style="text-align: center;">----</p>	1-5, 13-27, 29,30, 33-36
P,X	<p>MORGAN WILLIAM D ET AL: "Solution structure of an EGF module pair from the Plasmodium falciparum merozoite surface protein 1." JOURNAL OF MOLECULAR BIOLOGY, vol. 289, no. 1, 28 May 1999 (1999-05-28), pages 113-122, XP000957556 ISSN: 0022-2836 cited in the application see Materials and Methods</p> <p style="text-align: center;">----</p> <p style="text-align: center;">-/--</p>	28-30, 33-36

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 00/01558

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>AUFIERO B M ET AL: "Epitope mapping of mAbs that inhibit or block secondary processing of MSP142."  AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE,  vol. 61, no. 3 SUPPL., 1999, pages 345-346, XP000957837  48th Annual Meeting of the American Society of Tropical Medicine and Hygiene; Washington, D.C., USA; November 28-December 2, 1999  ISSN: 0002-9637  abstract</p> <p>-----</p>	1-4



**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

Continuation of Box I.1

Although claims 23-26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although claim 28 could be, at least partially, be considered as a mere presentation of information according to rule 39.1 PCT, the search has been carried out as far as possible in our systematic documentation.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 00/01558

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9730158 A	21-08-1997	FR 2744724 A	14-08-1997
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[Continued on next page]

(54) Title: VARIANT OF THE PLASMODIUM MEROZOITE SURFACE PROTEIN (MSP-1) AND VACCINE COMPRISING SAID VARIANT

<i>P. falciparum</i> domain-1	1	10	20	30	40
<i>P. falciparum</i> domain-2	NISQ-HQGVK---	KQGPNSGCEHLD---	ERECKCKHAY---	KQEGCKVENPNP---	
	50	60	70	80	90
	-----	TONEN-NGGQADAKCTEEDSGSNGKKITCPCTKPDSYPLEDGHFCSSEN-----			
<i>P. vivax</i> domain-1	TMSSEHTCIDT---	NVPDAAQRYLDGT---	EEWRCLTIS---	KEEGCKVPASNV---	
<i>P. vivax</i> domain-2	-----	TCKDN-NGGCAPEAEQMTDS---	NKIVCKCTREGSEPLEDGVFCSSSS-----		
legf human epidermal growth factor	--NSYPCGPPSSYDGYCLNGGVEMHIES---	LDSYTCGVIGY---	SGPCQTRDLRWELR		
EGF consensus	--C--	--C--	--C--	--C--	--C--
a = aromatic (Phe or Tyr)					
reduced core segment for alignment (14 aa)		XXXXX.....XXXXXXXXXX			

(57) Abstract: A non-naturally occurring variant of a C-terminal fragment of a *Plasmodium* merozoite surface protein-1 (MSP-1) wherein said variant has (i) a reduced affinity, compared with a naturally occurring *Plasmodium* MSP-1<sub>19</sub>, for at least one first antibody capable of blocking the binding of a second antibody, which second antibody inhibits the proteolytic cleavage of *Plasmodium* MSP-1<sub>42</sub> and (ii) substantially the same affinity for at least one third antibody compared with said naturally occurring *Plasmodium* MSP-1<sub>19</sub>, which third antibody inhibits the proteolytic cleavage of *Plasmodium* MSP-1<sub>42</sub> is provided for use in an anti-malarial vaccine.

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VARIANT OF THE PLASMODIUM MEROZOITE SURFACE PROTEIN (MSP-1) AND  
VACCINE COMPRISING SAID VARIANT

Field of the Invention

- 5 The present invention relates to modified *Plasmodium* MSP-1 protein variants and their use in producing a vaccine against malaria. It also relates to a method for the rational design of suitable variants.

Background to the Invention

10

Malaria is a devastating disease that causes widespread morbidity and mortality in areas where it is transmitted by anopheline mosquitoes. In areas of high transmission young children and non-immune visitors are most at risk from this disease, which is caused by protozoa of the genus *Plasmodium*. In areas of lower or unstable transmission, epidemics  
15 of the disease can result and afflict individuals of all ages. The most dangerous form of malaria, responsible for much of the morbidity and most of the mortality, is caused by the species *Plasmodium falciparum*. It has been estimated that 2 billion people are at risk from malaria, with 200-300 million clinical cases and 1-2 million deaths each year.

20

The parasite has a complex life cycle in its human and mosquito hosts. In humans the stage of the life cycle which is responsible for the clinical symptoms of the disease occurs in the bloodstream. During this phase the parasite is largely hidden within host red blood cells. Here the parasite grows and multiplies. For example, within a red blood cell each *P. falciparum* parasite divides several times to produce approximately 20 new ones during  
25 a 48 hour cycle. At this point the red blood cell is burst open and the parasites (called merozoites at this stage) are released into the bloodstream. The merozoites must enter new red blood cells in order to survive and for the cycle of replication in the blood to continue. If the parasites do not manage to enter red blood cells they cannot survive for very long and are rapidly destroyed. Symptoms of malaria such as fever are associated with this  
30 cyclic merozoite release and re-invasion of red blood cells.

There is an urgent need for a vaccine against malaria. There is no effective vaccine currently available. In addition, mosquito control by the spraying of residual insecticides is either becoming ineffective or considered to be unacceptable, and there is a very worrying spread of drug resistance within parasites. The rapid spread of drug resistance is  
5 worrying because compounds such as the cheap and once-effective chloroquine are no longer useful in many parts of the world, and there are few if any new drugs available that are both cheap and effective. Vaccines against microorganisms can be very cost effective and efficient ways to protect populations against infectious diseases.

10 Because of the complexity of the parasite's life cycle there are a number of points in its development within humans that could be the target of a protective immune response. It is known that with increasing age and exposure individuals do become immune to malaria, suggesting that protective responses do develop with time. Broadly speaking there are three types of vaccine strategy: to target the pre-erythrocytic stages, the asexual  
15 blood stage and the sexual stage. The pre-erythrocytic stages are the sporozoites that are injected by an infected mosquito when it takes a blood meal and the initial development of the parasite in the liver. The asexual blood stage is the infection and release of merozoites from red blood cells that occurs in a cyclic manner, and the stage responsible for the manifestation of the clinical symptoms. The sexual stage takes place in the mosquito's  
20 gut after it has ingested gametocytes in a blood meal and this initiates the infection of the insect to complete the cycle; a vaccine against the sexual stages would not protect the individual but could reduce transmission and therefore the incidence of malaria in a given human population.

25 During the asexual cycle in the blood the parasite is directly exposed to the host's immune system, and in particular to antibodies circulating within the bloodstream, only transiently: when merozoites are released by rupture of one cell and before they penetrate another. If there are specific antibodies that can bind to the surface of the parasite then it is possible that these antibodies will interfere with the ability of the parasite to invade a new red  
30 blood cell. In fact it has been shown that several monoclonal antibodies that recognise single epitopes on parasite surface proteins, are capable of neutralising the parasite and preventing the cycle of reproduction within red blood cells.

One of the best characterised proteins on the surface of the merozoite is called merozoite surface protein 1 (MSP-1). MSP-1 is a large protein that varies in size and amino acid sequence in different parasite lines. It is synthesised as a precursor molecule of ~200 kDa by the intracellular parasite and located on the parasite's surface. During release of merozoites from red blood cells and the re-invasion of new erythrocytes the protein undergoes at least two proteolytic modifications. In the first modification as a result of a process called primary processing, the precursor is cleaved to four fragments of ~83, 30, 38 and 42 kDa that remain together as a complex on the merozoite surface. This complex also contains two other proteins of 22 kDa and 36 kDa derived from different genes. The complex is maintained by non-covalent interactions between the different subunits and is held on the merozoite surface by a glycosyl phosphatidyl inositol anchor, attached to the C-terminus of the 42 kDa fragment and inserted into the plasma membrane of the merozoite. At the time of merozoite invasion of an erythrocyte the C-terminal 42 kDa fragment is cleaved by a second proteolytic cleavage in a process called secondary processing. The result of secondary processing is that the entire complex is shed from the surface of the merozoite except for a C-terminal sub-fragment that consists of just under one hundred amino acids and which is carried into the newly invaded erythrocyte on the surface of the merozoite.

Based on sequence similarities, the structure of this small C-terminal fragment (called MSP-1<sub>19</sub>) was suggested to consist of two epidermal growth factor (EGF)-like domains (see sequence in Figure 1) (Blackman *et al.*, 1991). An EGF-like motif consists of a 45-50 amino acid sequence with a characteristic disulphide bonding pattern and such domains occur frequently in extracellular modular proteins of animals. In the MSP-1 C-terminal fragment each of the motifs contains six Cys residues proposed to form three disulphide bonds and each motif has a partial match to the EGF consensus (see Figure 1). However, because the degree of similarity is limited and since the pattern of its disulphide bonding is not known, the designation of the MSP-1 C-terminal fragment as comprised of EGF-like structures has been regarded as tentative. Other relatively divergent potential EGF-like sequences occur in Plasmodium proteins, but previous structure determinations have been confined to those from metazoan organisms (Campbell *et al.*, 1998).

A number of studies have implicated MSP-1 as the target of a protective immune response. Although the goal of this work is to develop a malaria vaccine for use in humans, out of necessity most of this experimental work has been done either in model  
5 animal systems or *in vitro*. These include studies of the effect of specific antibodies on parasite invasion of erythrocytes *in vitro*, passive immunisation studies in rodent malaria models in laboratory mice and direct immunisation in both rodent and primate malaria models using either native protein (derived from the parasite) or recombinant protein expressed from parts of the MSP-1 gene in heterologous organisms. Sero-epidemiological  
10 studies have also showed a correlation between human antibody responses to parts of the MSP-1 molecule and protection against clinical disease. Much, but not all, of the work has focused on the immune response to the C-terminal MSP-1<sub>19</sub>. For example some monoclonal antibodies that recognise MSP-1<sub>19</sub> prevent red blood cell invasion in *in vitro* cultures (Blackman *et al.*, 1990). Interestingly, these antibodies that inhibit invasion also  
15 inhibit the secondary processing of the 42 kDa fragment, suggesting the mechanism by which they work is by steric hinderance of the protease responsible for secondary processing (Blackman *et al.*, 1994). Since secondary processing goes to completion during successful invasion, if it cannot occur then invasion is interrupted.

20 All of the work described above would suggest that MSP-1 and in particular polypeptides based on the C-terminal sequence that forms the 42 kDa or the MSP-1<sub>19</sub> region, should be very good candidates for malaria vaccine development. However, several studies have shown that the epitopes or binding sites for antibodies on MSP-1<sub>19</sub> require a correct polypeptide tertiary structure, and that this is destroyed by treatments that reduce the  
25 disulphide bonds that are postulated to be present between the cysteine residues present in MSP-1<sub>19</sub>. This limitation appears to have been overcome by the expression of recombinant protein in ways that allow antibodies that recognise the native parasite MSP-1 to bind. Other investigators have suggested that other parts of MSP-1 also have potential for inclusion in a vaccine, however the MSP-1 C-terminal fragment is currently  
30 the lead candidate for development of a vaccine against the blood stages of the malaria parasite (Diggs *et al.*, 1993; Stoute *et al.*, 1998).



As stated above, every ~48 hours *P. falciparum* merozoites are released from the infected erythrocyte to re-invade new red blood cells and during this time they are exposed to the host's immune system. Therefore, the question arises as to how the parasite has evolved to avoid the potentially lethal effects of, for example, neutralising antibodies. In other  
5 infectious micro-organisms it is clear that there is a constant battle between the immune system and the micro-organism, and that sophisticated mechanisms have been evolved by micro-organisms to evade the immune response. For example antigenic variation and antigenic diversity are two mechanisms that involve presenting the immune system with "a moving target" such that even though an immune response to one variant of the micro-  
10 organism may kill that variant, new variants are produced that are at least partially or fully resistant to the immune response. In the case of malaria merozoites and in particular MSP-1 an alternative mechanism has been proposed whereby the binding of some antibodies ("blocking antibodies") can prevent the binding of neutralising antibodies and thereby allow the parasite to successfully invade a red blood cell even in the presence of  
15 neutralising antibodies (Guevara Patiño *et al.*, 1997). These blocking antibodies may be of two types, those against epitopes that are formed from amino acids that are distant in the linear primary sequence from the epitopes that are the target of neutralising antibodies, and those that are against epitopes that overlap with the epitopes of the neutralising antibodies. This represents a novel mechanism by which a parasite can evade an effective  
20 immune response, and unlike mechanisms based on antigenic polymorphism or diversity, it is not dependent upon amino acid sequence diversity.

Some monoclonal antibodies (mAbs) that bind to MSP-1<sub>19</sub> inhibit the proteolytic cleavage and erythrocyte invasion, suggesting that cleavage is a prerequisite for invasion  
25 (Blackman *et al.*, 1994). Other mAbs that bind to the MSP-1 C-terminal fragment do not inhibit processing or invasion but block the binding of the inhibitory neutralizing antibodies. Other antibodies that bind to MSP-1<sub>19</sub> neither inhibit nor block the binding of inhibitory antibodies. In the presence of blocking antibodies, inhibitory antibodies are ineffective and invasion proceeds. The balance between inhibitory and blocking  
30 antibodies induced by immunisation may be a critical factor in determining whether or not the immune response is effective in preventing invasion (Guevara Patiño *et al.*, 1997).

### Summary of the Invention

An object of the present invention is therefore to provide an effective vaccine against the malaria parasite based on variants of the *Plasmodium* MSP-1 protein. In designing such a vaccine, the following criteria should be met:

1. The amino acid sequence of the polypeptide to be used in the vaccine should contain epitopes that are the targets of, and can induce, neutralising antibodies.

2. The polypeptide should ideally not include amino acid sequences that only form epitopes for blocking antibodies.

3. If the polypeptide contains epitopes for both neutralising and blocking antibodies then it should be modified to remove the blocking antibody epitopes without affecting the neutralising epitopes.

To assist in the design of candidate vaccine polypeptides fulfilling these three criteria, it is important to determine the three-dimensional structure of the MSP-1 C-terminal fragment since this will help in mapping sites of antibody interactions with this fragment. We have therefore determined the solution structure of the MSP-1 C-terminal, including the pattern of disulphide bonding, using NMR techniques.

We have made amino acid substitutions in the sequence of MSP-1<sub>19</sub> that prevent the binding of individual blocking monoclonal antibodies, without affecting the binding of neutralising antibodies. By determining the 3-dimensional structure of MSP-1<sub>19</sub> we have identified where these antibody binding sites are located in the tertiary structure and this has allowed other amino acid substitutions to be made that have similar properties. We have shown that several substitutions, each affecting the binding of one or more blocking antibodies can be combined into a single molecule, and that these modified molecules continue to bind the neutralising antibodies but fail to bind any of the blocking antibodies. Such modified molecules are expected to be much more effective than the natural or wild-type protein structure at inducing a protective neutralising antibody response when used to

immunise individuals as a malaria vaccine. In addition we have made other modifications in the primary structure of the molecule which do not affect the binding of the neutralising antibodies but which may contribute to increased immunogenicity of the molecule. The modified MSP-1<sub>19</sub> structures, either alone or coupled to other carriers, which may or may not contain other parts of MSP-1 to enhance the immunogenicity (for example a combination of the remainder of the MSP-1<sub>42</sub> kDa fragment with the modified MSP-1<sub>19</sub>) and provide additional T cell epitopes, would be more effective vaccines than equivalent structures that have not been modified in this way.

- 10 Accordingly, the present invention provides a non-naturally occurring variant of a C-terminal fragment of a *Plasmodium* merozoite surface protein-1 (MSP-1) wherein said variant has (i) a reduced affinity, compared with a naturally occurring *Plasmodium* MSP-1<sub>19</sub>, for at least one first antibody capable of blocking the binding of a second antibody, which second antibody inhibits the proteolytic cleavage of *Plasmodium* MSP-1<sub>42</sub> and (ii) substantially the same affinity for said second antibody compared with said naturally occurring *Plasmodium* MSP-1<sub>19</sub>.

Preferably, the *Plasmodium* MSP-1<sub>19</sub> and MSP-1<sub>42</sub> are *Plasmodium falciparum* MSP-1<sub>19</sub> and MSP-1<sub>42</sub>.

20 The first antibody is preferably selected from mAbs IE1, 2.2, 7.5, 9C8 and 111.4. The second antibody is preferably selected from mAbs 12.8, 12.10 and 5B1.

- 25 The present invention further provides a non-naturally occurring variant of a C-terminal fragment of a *Plasmodium* merozoite surface protein-1 (MSP-1) comprising an amino acid modification at any one of amino acid residues 14, 15, 27, 31, 34, 43, 48 and 53 of the *Plasmodium falciparum* MSP-1<sub>19</sub> amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

- 30 Preferably said modifications are substitutions selected from Gln14→Arg, Gln14→Gly, Asn15→Arg, Glu27→Tyr, Leu31→Arg, Tyr34→Ser, Tyr34→Ile, Glu43→Leu, Thr48→Lys and Asn53→Arg and their equivalents in other *Plasmodium* MSP-1<sub>19</sub>

polypeptides. More preferably said substitutions are combinations of substitutions selected from [Glu27→Tyr, Leu31→Arg and Glu43→Leu], [Glu27→Tyr, Leu31→Arg, Tyr34→Ser and Glu43→Leu], [Asn15→Arg, Glu27→Tyr, Leu31→Arg and Glu43→Leu] and their equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

5

In a preferred embodiment, a variant MSP-1 polypeptide of the invention further comprises a mutation at Cys12 and/or Cys28 of the *Plasmodium falciparum* MSP-1<sub>19</sub> amino acid sequence shown as SEQ I.D. No. 1. Preferably such modifications are substitutions selected from Cys12→Ile and Cys28→Trp, and Cys12→Ala and  
10 Cys28→Phe.

Most preferably the substitutions are combinations selected from [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Glu43→Leu], [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Glu43→Leu, Asn53→Arg],  
15 [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Tyr34→Ser, Glu43→Leu, Asn53→Arg] and their equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

The present invention also provides a method for producing a *Plasmodium* MSP-1 variant  
20 for use in preparing a vaccine composition which method comprises modifying one or more amino acid residues of a *Plasmodium* MSP-1 C-terminal fragment such that the resulting derivative has (i) a reduced affinity, compared with a naturally occurring *Plasmodium* MSP-1<sub>19</sub>, for at least one first antibody capable of blocking the binding of a second antibody, which second antibody inhibits the proteolytic cleavage of *Plasmodium*  
25 MSP-1<sub>42</sub> and (ii) substantially the same affinity for said second antibody compared with said naturally occurring *Plasmodium* MSP-1<sub>19</sub>. In particular the method of the invention preferably comprises as a preliminary step, selecting a candidate amino acid residue by reference to a three dimensional NMR model structure, preferably as set out in Table 2. More specifically, the 3D model structure is used to select a surface exposed amino acid  
30 residue. Advantageously, a further step is included of computer modelling the three dimensional structure of the variant to exclude polypeptides that do not fold correctly.

The present invention also provides a non-naturally occurring *Plasmodium* MSP-1 variant obtained by the method of the invention.

In a further aspect, the present invention provides a polynucleotide encoding a variant of the invention operably linked to a regulatory sequence capable of directing the expression of said nucleotide in a host cell. The polynucleotide may comprise a sequence which has been optimised for expression in the host cell. The host cell may be a *Pichia pastoris* cell. Also provided is a nucleic acid vector comprising a polynucleotide of the invention, including viral vectors, and a host cell comprising a nucleotide or vector of the invention.

In another aspect, the present invention provides a pharmaceutical composition comprising a variant of the invention, a polynucleotide of the invention or a vector of the invention together with a pharmaceutically acceptable carrier or diluent.

Preferably, the composition further comprises an immunogenic *Plasmodium* polypeptide or fragment or derivative thereof such as MSP-1<sub>33</sub> or a fragment or derivative thereof which may be covalently attached to the non-naturally occurring MSP-1<sub>19</sub>. It is preferred not to use wild-type MSP-1<sub>19</sub> sequences. The further immunogenic peptide may itself be derivatised in an analogous manner as described above for MSP-1<sub>19</sub>. Thus, epitopes present in the peptide may be identified and modified to prevent binding of blocking antibodies, without affecting the binding of neutralising antibodies. These epitopes may be capable of binding to antibodies which have similar properties to the first antibody described above, for example, in binding affinity. The further immunogenic peptide may comprise several such modifications in its amino acid sequence.

The present invention also provides a method for producing anti-MSP-1 antibodies which method comprises administering a polypeptide variant of the invention, or a polynucleotide of the invention or a vector of the invention to a mammal, typically a non-human mammal.

In a preferred embodiment, the present invention provides a method for producing polyclonal anti-MSP-1 antibodies which method comprises administering a polypeptide

variant of the invention, or a polynucleotide of the invention or a vector of the invention to a mammal, typically a non-human mammal, and extracting the serum from said mammal. Also provided is an antibody produced by the said methods.

- 5 The polypeptides, nucleotides and vectors of the present invention may be used in methods of treating and/or preventing malaria caused by *Plasmodium* species, in particular *Plasmodium falciparum*. Accordingly, the present invention provides a method of inducing immunity against malaria induced by *Plasmodium falciparum* which comprises administering to a person in need of such immunity an effective amount of a variant, a  
10 polynucleotide or a vector of the invention.

Also provided is a method of immunizing a mammal, said method comprising administering an effective amount of a variant, a polynucleotide or a vector of the invention. In particular, said mammal is immunized against malaria. Preferably the  
15 mammal is a human.

The present invention also provides a method of treating a malaria infection in a human patient which comprises administering to the patient an effective amount of the pharmaceutical composition of the invention.

20

We further provide according to the present invention a nucleic acid encoding a *Plasmodium* MSP-1 polypeptide, in which the nucleic acid is optimised for expression in a heterologous host cell. Preferably, the heterologous host is a *Pischia pastoris* cell. The MSP-1 polypeptide may be selected from the group comprising an MSP-1<sub>42</sub> polypeptide  
25 comprising a sequence shown in Figures 2C and 2E, an MSP-1<sub>19</sub> polypeptide comprising a sequence shown in Figure 2C, and an MSP-1<sub>33</sub> polypeptide comprising a sequence shown in Figure 2E. The optimised nucleic acid may comprise a sequence selected from the sequences of Figure 2A, Figure 2B and Figure 2D. We further provide a vector comprising such a nucleic acid, a host cell comprising such a vector, and a pharmaceutical  
30 composition comprising such a nucleic acid or a vector, together with a pharmaceutically acceptable carrier or diluent. The pharmaceutical composition may further comprise an immunogenic *Plasmodium* polypeptide or fragment or derivative thereof.

### Detailed description of the invention

Although in general the techniques mentioned herein are well known in the art, reference  
5 may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual  
(1989) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley &  
Sons, Inc.

#### A. MSP-1 variant polypeptides

10 The variant MSP-1 polypeptides of the present invention will be described with reference  
to *Plasmodium falciparum* MSP-1 amino acid sequences. However, it should be  
appreciated that except where otherwise stated, all references to MSP-1 polypeptides  
include homologues of MSP-1 found in other *Plasmodium* species, such as *P. vivax*,  
15 *P. malariae* and *P. ovale* which all infect humans and *P. yoelii* which infects mice.

The variant MSP-1 polypeptides of the present invention are based on C-terminal  
fragments of the *Plasmodium falciparum* MSP-1<sub>42</sub> polypeptide shown as SEQ I.D. Nos. 2  
or 3. Such polypeptides will comprise some or all of the MSP-1<sub>19</sub> region (SEQ I.D. No.  
20 1), preferably at least substantially all of the domain 1 and/or domain 2 EGF-like  
sequences found in MSP-1<sub>19</sub> (approximately amino acids 1-47 and amino acids 48-96,  
respectively, of SEQ I.D. No. 1). It is particularly preferred to use regions that are  
conserved in most, more preferably all parasites of a single species to increase the  
effectiveness of the variant as a vaccine against a wide range of strains.

25 Variant MSP-1 polypeptides of the present invention comprise modifications to their  
primary amino acid sequence that reduce the ability of blocking antibodies to bind to the  
MSP-1 polypeptides. In addition, any modifications made should maintain epitopes  
recognised by neutralising antibodies such that the affinity of the neutralising antibodies  
30 for the MSP-1 variant is substantially the same as for naturally-occurring MSP-1  
polypeptides (such as an MSP-1<sub>42</sub> polypeptide having the sequence shown in SEQ I.D.  
Nos. 2 or 3). Some reduction in the binding of some neutralising antibodies may be

tolerated since the primary objective is to inhibit the binding of blocking antibodies and it is likely that an effective reduction in the binding of blocking antibodies will compensate in terms of overall vaccine efficacy for a small reduction in neutralising antibody binding.

5 Neutralising antibodies in the context of the present invention are antibodies that inhibit malaria parasite replication. A variety of neutralising antibodies, polyclonal and monoclonal, are known in the art, including mAbs 12.8, 12.10 and 5B1 referred to in the Examples. The activity of neutralising antibodies can be determined in a variety of ways that have been described in the art. For example, a convenient assay method described in  
10 Blackman *et al.*, 1994 involves using preparations of merozoites (Blackman *et al.*, 1993; Mrema *et al.*, 1982) to measure cleavage of MSP-1<sub>42</sub> into MSP-1<sub>33</sub> and MSP-1<sub>19</sub>. Briefly, freshly isolated merozoites are washed in ice-cold buffer and divided up into aliquots of about  $2 \times 10^9$  merozoites. A test antibody is added to each aliquot and the sample incubated at 37°C for 1 hour. The samples are then subjected to SDS-PAGE under non-  
15 reducing conditions on a 12.5% polyacrylamide gel, Western blotted and the blot probed with antiserum to MSP-1<sub>33</sub>. In the control sample, two main bands are seen – one corresponding to MSP-1<sub>42</sub> and one lower molecular weight band corresponding to MSP-1<sub>33</sub>. Neutralising antibodies will reduce the amount of the lower molecular weight band as a result of inhibiting secondary proteolytic processing of MSP-1<sub>42</sub>.

20

This method is a particularly preferred method for assessing the efficacy of neutralising antibodies in the presence of antibodies believed to act as blocking antibodies. Where candidate competing blocking antibodies are to be tested, the merozoite sample is preincubated with a blocking antibody for 15 mins on ice prior to incubation with a  
25 neutralising antibody at 37°C for 1 hour as described above. Thus blocking antibodies can readily be identified and/or characterised using such an assay method.

Other assay methods include merozoite invasion inhibition tests as described in Blackman *et al.*, 1990.

30

As discussed above, blocking antibodies are defined in the context of the present invention as antibodies that inhibit the binding of neutralising antibodies to MSP-1 but



which do not themselves inhibit invasion of red blood cells by malaria parasites. Thus they “block” the neutralising function of the neutralising antibodies. A variety of blocking antibodies have been characterised in the art, including mAbs IE1, 2.2, 7.5 and 111.4 referred to in the Examples. As discussed above, blocking antibodies can conveniently be identified and/or characterised using assays that test their effect on neutralising antibody function.

Modifications that may be made to produce MSP-1 variants of the invention include substitutions, deletions and insertions. It is particularly preferred to use substitutions to minimise disruption of the secondary/tertiary structure of the polypeptide. Furthermore, particularly preferred substitutions are those that replace one class of amino acid with another class, such as an aliphatic non-polar residue with a charged polar residue. For example, the twenty naturally occurring amino acids may be divided into four main groups (aliphatic non-polar [G, A, P, I, L and V], polar un-charged [C, S, T, M, N and Q], polar charged [D, E, K and R] and aromatic [H, F, W and Y]) and it is preferred to replace an amino acid from one group with an amino acid from another group.

Other possibilities include replacing a positively charged side chain with a negatively charged side chain, replacing an amino acid with a large side chain with an amino acid with a smaller or no side chain (glycine), replacing a polar amino acid with a charged polar amino acid, replacing a large aromatic amino acid with an amino acid with a small side chain, and replacing cysteine residues that are involved in disulphide bonds.

Particularly preferred modifications are an amino acid modification at any one of amino acid residues 14, 15, 27, 31, 34, 43, 48 and 53 of the *Plasmodium falciparum* MSP-1<sub>19</sub> amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other *Plasmodium* MSP-1<sub>19</sub> polypeptides. These residues are all almost within the EGF-like domain 1. It is known that the epitopes of some antibodies contain amino acid sequences that are within EGF-like domain 2, therefore equivalent modifications may also be made in EGF-like domain 2. Preferred examples of modifications include the following substitutions Gln14→Arg, Gln14→Gly, Asn15→Arg, Glu27→Tyr, Leu31→Arg, Tyr34→Ser, Tyr34→Ile, Glu43→Leu, Thr48→Lys and/or Asn53→Arg and their equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

It is especially preferred to carry out more than one modification, i.e. to use combinations of modifications, such as two or more or three or more. In a preferred embodiment, an MSP-1 variant of the invention comprises a combination of amino acid substitutions selected from [Glu27→Tyr, Leu31→Arg and Glu43→Leu], [Glu27→Tyr, Leu31→Arg, Tyr34→Ser and Glu43→Leu], [Asn15→Arg, Glu27→Tyr, Leu31→Arg and Glu43→Leu] and their equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

A particularly preferred combination further comprises a modification to Cys12 and/or Cys28 (and/or their equivalent residues in EGF-like domain 2) to disrupt the disulphide bond. Preferably such modifications are substitutions selected from Cys12→Ile and Cys28→Trp, and Cys12→Ala and Cys28→Phe.

Most preferably the substitutions are combinations selected from [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Glu43→Leu], [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Glu43→Leu, Asn53→Arg], [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Tyr34→Ser, Glu43→Leu, Asn53→Arg] and their equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

Substitutions are not confined to using naturally occurring amino acids – non-naturally occurring amino acid analogues may also be used, in particular where solid phase synthesis is to be used to chemically synthesise the variant, as opposed to recombinant technology.

Modifications to MSP-1 amino acid sequences may be carried out using standard techniques such as site-directed mutagenesis using the polymerase chain reaction. Alternatively, variants may be obtained by solid phase synthetic techniques.

To determine whether a variant MSP-1 polypeptide produced by modification of its primary amino acid sequence complies with the criteria specified above, the affinity of at least one neutralising antibody and at least one blocking antibody for the variant

polypeptide compared with the naturally occurring MSP-1 sequence may be tested. Ideally more than one of each type of antibody should be used, for example two or three.

The ability of antibodies to bind to the variant and wild-type polypeptides may be determined using any one of a variety of methods available in the art for determining antibody-epitope binding. One such method, described in the Examples, involves the use of MSP-1 sequences expressed as fusion proteins with a protein tag such as glutathione-S-transferase (GST). These GST-fusion proteins are typically immobilised to a solid phase such as glutathione sepharose beads or a BIAcore sensor chip. Binding of antibodies, such as monoclonal antibodies, to the fusion proteins may be determined using standard techniques such as Western blotting and/or by labelling the antibodies with a radioactive label such as  $^{125}\text{I}$ . The use of BIAcore technology allows easy quantitation of the results.

Preferably, the reduction in binding of at least one of the blocking antibodies tested is at least 50% compared to wild-type MSP-1, more preferably at least 75, 80 or 90%, typically as assessed using recombinantly expressed MSP-1 immobilised to a BIAcore sensor chip. By contrast, the binding of at least one, for example at least two or three, of the neutralising antibodies tested, more preferably at least half of the neutralising antibodies tested, more preferably substantially all of the neutralising antibodies tested is reduced by less than 50%, more preferably less than 25%. The number of neutralising antibodies that need be tested to confirm compliance with the test criteria will not typically exceed from three to five different antibodies (three antibodies are used in the Examples). In a particularly preferred embodiment the binding of at least one neutralising antibody is increased by at least 10%.

The results given in Table 2 in the Examples provide partial guidance to the skilled person as to which residues may be modified to produce a variant MSP-1 of the invention. However, the provision herein for the first time of the three dimensional solution structure of MSP-1<sub>19</sub> provides the skilled person with further detailed guidance as to which residues may be altered. In particular, epitopes are expected to be exposed to the aqueous environment on the exterior of the MSP-1<sub>19</sub> fragment. Consequently, the precise structural information provided which teaches the position of surface exposed amino acids

allows the skilled person to target those residues for modification. This data is given in Tables A/B and has also been submitted to the Protein Data Bank (PDB Accession no. 1CEJ). It enables the skilled person to identify the precise location of individual amino acids in the three dimensional structure. Typically, the data is loaded into suitable software, well-known in the art such as Insight II, MOLSCRIPT GRAS P and RASMOL.

Further, knowing the location of a modification in the 3-dimensional structure which affects the binding of a blocking antibody without affecting the binding of the neutralising antibodies, it is possible to identify other residues that are on the surface and in the vicinity of the original modification and which may be easily modified to further improve the properties of a modified protein. These residues may be in either the first or the second EGF-like motifs or in the sequence between them. Since it is known that an antibody binding site can encompass a volume that corresponds approximately to the range of 5 to 8 amino acids, it is clear that modifications of these adjacent residues may also affect the affinity of the protein for the blocking antibodies. Once an adjacent amino acid has been identified it can be modified according to the principles outlined above and the contribution of the modification to the overall antigenicity and immunogenicity of the protein, either alone or in combination with other modifications, can be assessed. Those changes that contribute to a reduced affinity for the blocking antibodies, without a substantial affect on binding of the neutralising antibodies can be incorporated into the improved protein. This can be a reiterative process.

In addition, the 3D NMR structure will enable the skilled person to carry out preliminary computer modelling studies of MSP-1<sub>19</sub> variants with specific modifications so that, for example variants that cannot fold properly may be discarded. This will assist in minimising the number of candidate MSP-1<sub>19</sub> variants that need be tested.

Thus the present invention also provides a computer readable medium having stored thereon a model of the MSP-1<sub>19</sub> NMR structure. In a preferred embodiment, said model is built from all or some of the NMR data shown in Tables A and B.

Variants of the present invention may optionally include additional MSP-1 sequences, in

particular regions of the MSP-1<sub>33</sub> region of MSP-1<sub>42</sub> to confer additional immunogenicity to the variant. Furthermore, additional sequences known to contain and promote T cell responses are advantageously included (i.e. T cell epitopes). Other modifications may also be made that increase immunogenicity such as modifications that alter the pathway of antigen processing and presentation.

Polypeptide variants of the invention are typically made by recombinant means, for example as described below. However they may also be made by synthetic means using techniques well known to skilled persons such as solid phase synthesis. Proteins of the invention may also be produced as fusion proteins, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and  $\beta$ -galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the immunogenicity of the MSP-1 variant.

Polypeptides of the invention may be in a substantially isolated form. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as substantially isolated. A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the polypeptide in the preparation is a polypeptide of the invention.

#### B. Polynucleotides and vectors

As discussed above, the variants of the present invention may be produced recombinantly using standard techniques. Thus, the present invention also provides a polynucleotide encoding a polypeptide MSP-1 variant of the invention. Polynucleotides of the invention may comprise DNA or RNA. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to

oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art.

5 Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of the invention. It will be understood by a skilled person that numerous different polynucleotides can encode the same polypeptide as a result of the degeneracy of the genetic code.

10 Polynucleotides of the invention comprise can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host  
15 cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect Sf9 cells. The host cell may be a methylotrophic yeast such as *Pichia pastoris*.

20 The coding sequence of natural or variant MSP polypeptides (including the polypeptide of the invention) may be modified for optimal expression in a host cell. For example, secondary modification such as N-glycosylation may be prevented by removal of sequences necessary for such modification. The sequence of the polypeptide may alternatively or in addition be modified with respect to codon usage for optimal expression  
25 in the host cell. Methods of mutagenising a sequence are known in the art; alternatively, the modified coding sequence may be generated by means of PCR gene assembly using overlapping synthetic oligonucleotides (Stemmer et al., 1995; Withers-Martinez et al., 1999).

30 Preferably, a polynucleotide of the invention in a vector is operably linked to a regulatory sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a

juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

5

Such vectors may be transformed or transfected into a suitable host cell using standard techniques above to provide for expression of a polypeptide of the invention. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions to provide for expression by the vector of a coding  
10 sequence encoding the polypeptides, and optionally recovering the expressed polypeptides.

15

The vectors may be for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable  
marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell. The vector may also be adapted to be used *in vivo*, for example in a method of gene therapy.

20

Promoters/enhancers and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. For example, prokaryotic promoters may be used, in particular those suitable for use in *E. coli* strains (such as *E. coli* HB101 or DH5 $\alpha$ ).

25

When expression of the polypeptides of the invention is carried out in mammalian cells, either *in vitro* or *in vivo*, mammalian promoters may be used. Tissue-specific promoters may also be used. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the promoter of the Rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE  
30 promoter, herpes simplex virus promoters or adenovirus promoters. All these promoters are readily available in the art.

### C. Administration

5 The variant MSP-1 polypeptides of the present invention and nucleic acid molecules may be used to treat or prevent malaria in animals, specifically humans.

10 The polypeptides of the invention may be administered by direct injection. Preferably the polypeptides are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration. Typically, each polypeptide is administered at a dose of from 0.01 to 30 µg/kg body weight, preferably from 0.1 to 10 µg/kg, more preferably from 0.1 to 1 µg/kg body weight. It is also possible to use antibodies prepared using the polypeptides of the invention, as described below, in treating or preventing *Plasmodium* infection. Neutralising antibodies, or fragments thereof which retain specificity for *Plasmodium* antigens, can be administered in a similar manner to the polypeptides of the invention.

20 The polynucleotides of the invention may be administered directly as a naked nucleic acid construct. When the expression cassette is administered as a naked nucleic acid, the amount of nucleic acid administered is typically in the range of from 1 µg to 10 mg, preferably from 100 µg to 1 mg.

25 Uptake of naked nucleic acid constructs by mammalian cells is enhanced by several known transfection techniques for example those including the use of transfection agents. Example of these agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectam<sup>TM</sup> and transfectam<sup>TM</sup>). Typically, nucleic acid constructs are mixed with the transfection agent to produce a composition.

30



Alternatively, the polynucleotide may be administered as part of a nucleic acid vector, including a plasmid vector or viral vector, such as a vaccinia virus vector. When the polynucleotide of the invention is delivered to cells by a viral vector of the invention, the amount of virus administered is in the range of from  $10^3$  to  $10^{10}$  pfu, preferably from  $10^5$  to  $10^8$  pfu, more preferably from  $10^6$  to  $10^7$  pfu. When injected, typically 1-10  $\mu$ l of virus in a pharmaceutically acceptable suitable carrier or diluent is administered.

Preferably the delivery vehicle (i.e. naked nucleic acid construct or viral vector comprising the polynucleotide for example) is combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

#### D. Preparation of Vaccines

Vaccines may be prepared from one or more polypeptides of the invention. They may also include one or more immunogenic *Plasmodium* polypeptides known in the art. Thus a vaccine of the invention may comprise one or more polypeptides of the invention and optionally, one or more polypeptides selected from, for example, the asexual blood stage proteins: apical merozoite antigen-1, erythrocyte binding antigen 175, erythrocyte membrane protein-1; the hepatic stage proteins: liver stage antigens 1 and 3; the sporozoite stage proteins: circumsporozoite protein, thrombospondin related adhesive protein; and the sexual stage proteins Pfs25 and Pfs28 polypeptides and immunogenic fragments thereof. Preferably, the other immunogenic *Plasmodium* polypeptides known in the art do not contain wild type MSP-1<sub>19</sub> sequences.

The preparation of vaccines which contain an immunogenic polypeptide(s) as active

ingredient(s), is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion. The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic polypeptide containing an MSP-1 antigenic sequence resulting from administration of this polypeptide in vaccines which are also comprised of the various adjuvants.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1% to 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%. Where the vaccine composition is

lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is preferably effected in buffer.

5 Capsules, tablets and pills for oral administration to a patient may be provided with an enteric coating comprising, for example, Eudragit "S", Eudragit "L", cellulose acetate, cellulose acetate phthalate or hydroxypropylmethyl cellulose.

10 The polypeptides of the invention may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric and maleic. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino  
15 ethanol, histidine and procaine.

#### E. Dosage and Administration of Vaccines

20 The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The quantity to be administered, which is generally in the range of 5 µg to 250 µg of antigen per dose, depends on the subject to be treated, capacity of the subject's immune system to synthesise antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered may depend on the judgement of the practitioner and may be  
25 peculiar to each subject.

The vaccine may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals  
30 required to maintain and or reinforce the immune response, for example, at 1 to 4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the need of the individual and be

dependent upon the judgement of the practitioner.

In addition, the vaccine containing the immunogenic MSP-1 antigen(s) may be administered in conjunction with other immunoregulatory agents, for example,  
5 immunoglobulins.

F. Preparation of antibodies against the polypeptides of the invention

The variant MSP-1 polypeptides prepared as described above can be used to produce  
10 antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide bearing an MSP-1 epitope(s). Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to an MSP-1 epitope contains antibodies to other antigens, the  
15 polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art.

Monoclonal antibodies directed against MSP-1 epitopes in the polypeptides of the invention can also be readily produced by one skilled in the art. The general methodology  
20 for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against MSP-1 epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

25 The polypeptides of the invention can also be used to select for human monoclonal antibodies using the variable regions of immunoglobulin heavy and light chains cloned in the form of a phage display library, preferably from individuals who have been previously exposed to a natural malaria infection.

30 Antibodies, both monoclonal and polyclonal, which are directed against MSP-1 epitopes are particularly useful in diagnosis, and those which are neutralising are useful in passive

immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotypic antibodies. Anti-idiotypic antibodies are immunoglobulins which carry an "internal image" of the antigen of the infectious agent against which protection is desired.

5 Techniques for raising anti-idiotypic antibodies are known in the art. These anti-idiotypic antibodies may also be useful for treatment of *Plasmodium* infections, as well as for an elucidation of the immunogenic regions of MSP-1 antigens. It is also possible to use fragments of the antibodies described above, for example, F(ab')<sub>2</sub>, Fab, Facb and scFv fragments.

10

It should be appreciated that features from various sections, aspects and embodiments of the invention as described above are generally equally applicable to other sections, aspects and embodiments *mutatis mutandis*.

15

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention. The Examples refer to the Figures. In the Figures:

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#### Detailed Description of the Figures

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Figure 1 - MSP-1 sequences aligned according to the EGF-like motif consensus. Top sequence: *P. falciparum* (SWISS-PROT MSP1 PLAFW). Second sequence: *P. vivax* Belem strain (PIR A45604). Third sequence: human EGF (PDB 1egf). Fourth sequence: EGF-like domain consensus (Prosite EGF1). Bottom sequence: 14 residue EGF core region used for structure alignment in Figure 6. Black highlighting indicates conserved residues of the EGF-like domain. Dark shading shows hydrophobic residues at the EGF-module pair interface in the *P. falciparum*, and corresponding conserved residues in the *P. vivax* sequence.

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Figure 2 - Sample of multidimensional heteronuclear NOESY experiments showing planes containing NOE connections to the MSP-1 C-terminal fragment Lys35 NH proton. Top: <sup>13</sup>C (D4) and <sup>1</sup>H(D3) plane from the 4D-[<sup>13</sup>C]-HMQC-NOESY-[<sup>15</sup>N]-HSQC

experiment, taken at the chemical shift values of Lys35 NH in  $^{15}\text{N}$ (D2) and  $^1\text{H}$ (D1). Bottom: strip from the 3D [ $^{15}\text{N}$ ]-NOESY-HSQC at the  $^1\text{H}$  chemical shift value of Lys35 NH (vertical axis, D1) taken at the plane of its  $^{15}\text{N}$  (D3) value. The horizontal  $^1\text{H}$  axis is aligned with that of the top spectrum. The weak cross-peaks at 2.72 and 3.01 ppm in the

5 3D spectrum do not show corresponding cross-peaks in the 4D spectrum because of the lower signal-to-noise ratio in the latter. These peaks have been assigned as the cross-peaks between Lys35 NH and Asn44  $\text{H}_{\beta 2}$  (2.72 ppm), and Cys30  $\text{H}_{\beta 3}$  and/or Cys41  $\text{H}_{\beta 2}$  (3.01 ppm).

10 Figure 3 - Stereo drawing showing the backbone C, N,  $\text{C}_\alpha$  atoms of the 32 refined structures in the final ensemble. The domain-1 is on the left (red), with domain-2 on the right (green), and both the N- and C-termini are near the bottom.

Figure 4 - MOLSCRIPT picture of the most representative model of the ensemble,

15 showing the backbone  $\text{C}_\alpha$  trace, antiparallel  $\beta$ -sheet elements, and disulphide bridges ( $\text{S}_\gamma$  atoms in yellow). Domain-1, red; Domain-2, green.

Figure 5 - Alignment of typical EGF-like family members with the fitpdb program, using the 14 amino acid "reduced core" consensus (Bersch *et al.*, 1998) (see Figure 1). The

20 aligned backbone segment in each structure is white. The structures are aligned relative to the most representative structure of the group (factor Xa), with increasing divergence from left to right. Numbers indicate the rmsd value of the aligned C, N,  $\text{C}_\alpha$  atoms. PDB identification codes: factor Xa (crystal structure), 1hcg; Complement C1r component, 1apq (14<sup>th</sup> model); human EGF, 1egf (11<sup>th</sup> model); fibrillin-1, domains-32 and -33, 1emn (minimized average structure); transforming growth factor- $\alpha$ , 2tgf (minimized average structure); MSP-1 domains-1 and -2, this study.

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Figure 6 - Backbone ribbon view of fibrillin-1 versus MSP-1 EGF module pair arrangements. Fibrillin-1 (1emn) cyan (domain-32) and magenta (domain-33) (Downing

30 *et al.*, 1996); MSP-1 domain-1 (yellow) and domain-2 (green). Structures were aligned as in Figure 6 by the core consensus of the N-terminal domain of each pair. The bound  $\text{Ca}^{2+}$  ions in the fibrillin-1 structure are shown as magenta spheres.

Figure 7 - Two views, a and b, (rotated 180° about the y-axis) of the electrostatic potential surface of the MSP-1 EGF module pair, calculated with GRASP. Red indicates negative charge, blue indicates positive charge, and white is neutral. The orientation of the views is shown by the adjacent worm diagrams.

Figure 8 - CPK model of the MSP-1 C-terminal fragment, showing the location of some mutations that affect binding of monoclonal antibodies. Domain-1 is towards the top and right sides, and domain-2 towards the bottom left.

Figure 9 - Examples of the binding of monoclonal antibodies to GST-MSP-1<sub>19</sub> detected by Western blotting. The binding of each monoclonal antibody to protein based on the wild type sequence and to proteins containing modified sequences is shown. The monoclonal antibodies are shown across the top. On the left is shown the proteins: WT, wild type sequence; 22, Leu22 to Arg; 26, Glu26 to Ile; 15, Asn15 to Arg; 27, Glu27 to Tyr; 31, Leu31 to Arg; 43, Glu43 to Leu; 27+31+43, Glu27 to Tyr and Leu31 to Arg and Glu43 to Leu; 15+27+31+43, Asn15 to Arg and Glu27 to Tyr and Leu31 to Arg and Glu43 to Leu.

Figure 10 - The binding of monoclonal antibodies to GST-MSP-1<sub>19</sub> detected by BIAcore analysis. The binding of each monoclonal antibody is normalised to 100% binding to protein based on the wild type sequence and the binding of proteins containing modified sequences is expressed as a percentage of this. WT, wild type sequence; 15, Asn 15→Arg; 26, Glu26→Ile; 27, Glu27→Tyr; 31, Leu31→Arg; 34, Tyr34→Ser; 43 Glu43→Leu.

Figure 11 - The binding of monoclonal antibodies to GST-MSP-1<sub>19</sub> containing multiple modifications detected by BIAcore analysis. The binding of each monoclonal antibody is normalised to 100% binding to protein based on the wild type sequence and the binding of proteins containing modified sequences is expressed as a percentage of this. WT, wild type sequence; The combinations contain 3 mutations [27+31+43], or 4 mutations ([27+31+34+43] and [15+27+31+43]), at each site the changes are those identified in Figure 10.

Figure 12 – Identification of blocking antibodies using a competitive binding assay and immobilised wild type GST-MSP-1<sub>19</sub>. The ability of antibodies to compete with the binding of mAbs 12.8 and 12.10 to GST-MSP-1<sub>19</sub> was measured using BIAcore analysis. Individual antibodies (x-axis) were bound to the antigen and then the amount of either  
 5 12.8 or 12.10 (inhibitory mAb) that could subsequently bind was quantified. The amount of binding is presented as a percentage of the total amount of either 12.8 or 12.10 bound in the absence of pre-incubation with another antibody.

Figure 13 - Antibodies induced by immunisation with a modified recombinant MSP-1<sub>19</sub> assayed for their ability to inhibit secondary processing. Washed 3D7 merozoites were either analysed directly without incubation (0 h) or incubated for 1 hour at 37°C in the presence of no serum (no serum), 1 mM PMSF as a control for complete inhibition, normal rabbit sera (normal serum), or serum from a rabbit immunised with the 15+27+31+43 modified protein (immune serum), all at 1:10 dilution in reaction buffer.  
 15 The level of MSP-1<sub>33</sub> released into the supernatant as a results of secondary processing was measured using an ELISA method and is represented by Absorbance at 492nm.

Figure 14. *Pichia pastoris* codon preference table used for input to the CODOP program.

20 Figure 15. DNA and protein sequences for the optimized synthetic MSP-142 gene. A: Complete sequence designed for optimum codon usage and expression in *P. pastoris*. B: Sequence of the synthetic MSP-119 construct in the expression vector pPIC9K-HXa. Uppercase letters: vector sequences, including the His<sub>6</sub> tag and factor Xa cleavage site (IEGR). Lowercase letters: synthetic MSP-119 coding sequence. The cloned sequence in  
 25 located at the SnaBI restriction site of the pPIC9K sequence. C: Expressed protein sequence of the synthetic MSP-119 construct. The sequence shown is produced as a fusion to the pPIC9K  $\alpha$ -factor secretion signal, following the kex2/STE13 processing sites. The synthetic MSP-119 is in bold-face type. D: Sequence of the MSP-133 construct. The cloned sequence is located at the SmaI site of the pUC118 vector. E: Predicted  
 30 protein sequence of the synthetic MSP-133 construct translation product.

Figure 16. Gene assembly PCR reactions for the MSP-133 and MSP-119 sequences. Reaction 1: 10  $\mu$ L aliquots of the assembly reactions. Reaction 2: 20  $\mu$ L aliquots of the



amplification reactions. The N-terminal and middle fragments were subsequently spliced together to form the MSP-133 synthetic construct. The C-terminal fragment synthesis reactions produced the optimized MSP-119 construct.

- 5 Figure 17. Expression of the synthetic MSP-119 protein in *P. pastoris*. Lanes 1-6: trichloroacetic acid precipitates of secreted recombinant protein from culture supernatants, without further purification (5  $\mu$ L each). Samples from duplicate cultures of three independent transformants. Lane 8,9: purified, deglycosylated MSP-119 produced from the original *P. falciparum* sequence. Lane 7,10: NOVEX molecular weight markers.

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Figure 18. A:  $\{^1\text{H}/^{15}\text{N}\}$ -HSQC spectrum of the protein (2.5 mM) expressed from the optimized synthetic MSP-119 gene. B: Control  $\{^1\text{H}/^{15}\text{N}\}$ -HSQC of deglycosylated protein (2.2 mM) expressed from the original *P. falciparum* sequence (Morgan et al., 1999).

## 15 Examples

### **Materials and Methods**

#### Protein expression and stable-isotope labelling for NMR

20

The coding sequence of the MSP-1 C-terminal fragment was cloned by polymerase chain reaction with Vent polymerase (New England Biolabs) from a plasmid containing the *Plasmodium falciparum* strain T9/94 fragment (Blackman et al., 1991), using primers that included codons for a 6 residue N-terminal His tag (CACCATCATCATCAC), and  
25 inserted into the *Sna*BI restriction site of the pPIC9K vector (Invitrogen). The sequence corresponds to residues 1526-1621 of the SWISS-PROT entry MSP1 PLAFW (accession number P04933). This produced an  $\alpha$ -factor fusion protein with the sequence ...KR/EA/EA/YHHHHHHNISQ....SSSN, where the slashes indicate kex2 and STE13 processing sites. High copy number transformants of the methylotrophic yeast  
30 *Komagataella (Pichia) pastoris* protease-deficient strain SMD1168 (*his4 pep4*) were isolated by screening for high G418 resistance (Clare et al., 1995).

A Mut<sup>+</sup> transformant was grown at 29.4 °C in a shaker-incubator in buffered minimal

medium (100 mM potassium phosphate, pH 6.0, yeast nitrogen base (0.34 % w/vol) (DIFCO: YNB without amino acids and without  $(\text{NH}_4)_2\text{SO}_4$ ), biotin ( $4 \times 10^{-5}$  % w/vol), Sigma antifoam 289 (0.01 % vol/vol), and carbon and nitrogen sources as described below. Unlabelled samples were initially grown in medium containing 1 % w/vol  $(\text{NH}_4)_2\text{SO}_4$  and  
5 1 % w/vol glycerol, and induced by transfer to medium containing 0.5 %  $\text{CH}_3\text{OH}$  as the carbon source. Labelled samples were initially grown in medium containing 0.2 % w/vol  $[\text{^{15}N}]-(\text{NH}_4)_2\text{SO}_4$  (Isotech), and 0.5 % w/vol glucose or  $[\text{^{13}C}_6]$ -glucose (Isotech), and induced by transfer to medium containing as carbon source 0.5 % w/vol  $\text{CH}_3\text{OH}$  or  $[\text{^{13}C}]$ - $\text{CH}_3\text{OH}$  (Isotech). The initial cultures were grown in 150 ml to a density of  $\sim 10 \text{ OD}_{600}$ ,  
10 then harvested and resuspended in methanol medium at 1  $\text{OD}_{600}$  in a volume of 1.5 L. Methanol-induced cultures were grown for 4 d, with daily addition of 7.5 ml  $\text{CH}_3\text{OH}$  or  $[\text{^{13}C}]$ - $\text{CH}_3\text{OH}$ , to a final density of  $\sim 18 \text{ OD}_{600}$ . This protocol produced a maximum yield of 24 mg/L of purified,  $^{13}\text{C}/^{15}\text{N}$  uniformly labelled protein at the final stage (see below). The YNB-based medium produced about 3-fold higher yields than the FM22 medium  
15 (Laroche *et al.*, 1994), for stable-isotope labeling of the MSP-1 C-terminal fragment.

Cells were removed by low-speed centrifugation, protease inhibitors added (COMPLETE<sup>TM</sup> tablets, Boehringer-Mannheim; 1 tablet/500 ml supernatant), and the supernatant was filter-sterilized. The supernatant was concentrated  $\sim 20$ -fold by  
20 ultrafiltration in a stirred cell (Amicon, YM3 membrane) at 4 °C. The pH was adjusted to 7.25 with KOH, and the partially N-glycosylated MSP-1 fragment was deglycosylated for 72 h at 37 °C with 5000 U PNGaseF (New England Biolabs). The carbohydrate was completely removed (as shown by electrophoresis and mass spectrometry), with the Asn 1 residue presumably converted to Asp in the process. The supernatant was clarified by low-  
25 speed centrifugation, 5 M NaCl was added to a final concentration of 0.3 M, and the sample was applied to a 2 ml Ni-NTA affinity column (QIAGEN), washed, and eluted with 250 mM imidazole according to manufacturer's instructions. The eluate was dialyzed against 50 mM sodium phosphate (pH 6.5), 50 mM NaCl, and then passed through a 1 ml Hi-Trap Q anion exchange resin (Pharmacia) to remove misfolded MSP-1  
30 that bound to the column. The MSP-1 fragment was characterized by Western blotting and electrospray mass spectrometry (data not shown). Two principal species of mass 11607 and 11807 Da were observed corresponding to the expected fragment, as well as a

fragment with an additional N-terminal Glu - Ala dipeptide resulting from incomplete STE13 processing of the  $\alpha$ -factor secretion signal.

Samples for NMR experiments were prepared in either 90 % H<sub>2</sub>O/10 % D<sub>2</sub>O with 0.01 % w/vol NaN<sub>3</sub>, or 100 % D<sub>2</sub>O, 50 mM sodium phosphate, 100 mM NaCl at pH 6.5, (pH uncorrected for deuterium isotope effects), at a concentration of 2.1 to 2.6 mM in 0.6 ml. Protein concentration was measured by UV absorbance at 280 nm, using a calculated molar extinction coefficient of 5220 liter mol<sup>-1</sup> cm<sup>-1</sup>. The protein was demonstrated to be monomeric by equilibrium ultracentrifugation of a 0.12 mM sample in the above buffer at 293 K.

#### NMR experiments and data processing

Most of the experiments were performed at 298 K, using Varian Unity and Unity-Plus spectrometers operating at 600 MHz and 500 MHz respectively. Details of the multidimensional experiments (Clore & Gronenborn, 1998) and acquisition parameters used for resonance assignments and structure determination are given in Tables A/B and have been submitted to the Protein Data Bank database (PDB Accession No 1CEJ).

All spectra were processed using Felix 95.0 or 97.0 (Biosym/MSI) using a 90 degree- or 72 degree-shifted sinebell-squared window function. Dimensions, zero-filling, and linear prediction details are summarized in Tables A/B and in the submission to the BioMagResBank. Four dimensional and interleaved spectra were processed in Felix using macros written in-house.

*Signal assignments:* Sequential assignments were made based on connectivities established primarily by CBCA(CO)NH and CBCANH experiments on uniformly <sup>13</sup>C/<sup>15</sup>N labelled protein. Side chain-spin system assignments were made on the basis of data from <sup>13</sup>C/<sup>1</sup>H -HCCH-TOCSY experiment correlated with information from <sup>15</sup>N/<sup>1</sup>H-TOCSY-HSQC and <sup>15</sup>N/<sup>1</sup>H-NOESY-HSQC, and HNHA and HNHB experiments. Assignments were obtained for <sup>1</sup>H, <sup>15</sup>N and aliphatic <sup>13</sup>C signals for 98% of side-chains and 96% of backbone amide groups. The list of assignments is given in Tables A/B and in the

submission to the Protein Data Bank database (PDB Accession No 1CEJ). The  $^{15}\text{N}\{^1\text{H}\}$  heteronuclear NOE experiment was carried out as described previously (Kay *et al.*, 1989; Polshakov *et al.*, 1997).

5 *Distance Restraints:* NOE- and ROE-derived distance restraints between backbone and side chain amide protons were obtained primarily from the 3D  $^{15}\text{N}$ -NOESY-HSQC,  $^{15}\text{N}$ -ROESY-HSQC, and 4D  $^{13}\text{C}$ -HMQC-NOESY- $^{15}\text{N}$ -HSQC experiments. Aliphatic to aliphatic proton distance restraints were obtained from a 4D  $^{13}\text{C}$ -HMQC-NOESY- $^{13}\text{C}$ -HSQC experiment. A 3D  $^{13}\text{C}$ -HMQC-NOESY experiment in  $\text{D}_2\text{O}$  was used to identify  
10 aliphatic to aromatic proton NOEs and 2D NOESY experiments were used to measure aromatic to aromatic proton NOEs. Crosspeaks were quantified by volume integration in Felix for 2D and 3D experiments and for the 4D  $^{13}\text{C}$ -HMQC-NOESY- $^{15}\text{N}$ -HSQC experiment, and from peak height measurements in the 4D  $^{13}\text{C}$ -HMQC-NOESY- $^{13}\text{C}$ -HMQC spectra. Crosspeaks were classified as strong, medium and weak and these were  
15 assigned to distance restraints of 0 - 2.8, 0 - 3.6, and 0 - 5.5 Å. Restraints from backbone amide signals were initially treated in this manner, and then recalibrated more precisely using 3D- $^{15}\text{N}$ -ROESY-HSQC data into four classes involving maximum distances of 2.6, 3.1, 3.6, and 4.1 Å. Restraints to groups of equivalent or non-stereoassigned protons were treated by  $r^{-6}$  summation. Most intraresidue distances ( $\text{HN-H}_\beta$  and  $\text{H}_\alpha\text{-H}_\beta$ ) were converted  
20 to  $\chi_1$  angle restraints as described below and these distance restraints were not included in the final list.

*Dihedral Angle Restraints:*  $\chi_1$  angles and stereospecific assignments of  $\beta$ -methylene protons were obtained using the grid-search program AngleSearch, with coupling constant  
25 and intraresidue ROE distance information (Polshakov *et al.*, 1995). The coupling constant information was provided by HNHB and HN(CO)HB spectral intensities for  $^3\text{J}(\text{HN-H}_\beta)$  and  $^3\text{J}(\text{CO-H}_\beta)$ , and intraresidue distances ( $\text{HN-H}_\beta$ ,  $\text{H}_\alpha\text{-H}_\beta$ ) were obtained from 3D  $^{15}\text{N}$ -ROESY-HSQC and 2D ROESY ( $\text{D}_2\text{O}$ ) experiments.  $^3\text{J}(\text{HN-H}_\alpha)$  coupling constants were obtained from the HNHA experiment. Residues with positive  $\phi$  angles (ca.  
30 +60 degrees) were identified by large intraresidue  $\text{H}_\alpha$  crosspeak intensities in the HN(CO)HB experiment, and  $\gamma$  angles near -60° degrees from strong  $\text{H}_{\alpha(i-1)}$  crosspeaks in the HNHB experiment. Ile and Leu  $\chi_2$  angles and Leu  $\delta$  stereoassignments were derived

from the LRCH experiment. Minimum ranges of 40 degrees ( $\chi_1, \chi_2$ ) and 50 degrees ( $\phi, \psi$ ) were used to account for errors and local dynamic effects on the coupling constants.

*Disulphide Bonding Pattern:* An initial set of 20 structures was calculated by simulated annealing using approximately 550 unambiguous NOE-derived distance restraints and 36  $\chi_1$  and  $\phi$  dihedral angle restraints but with no hydrogen bonding or disulphide bond constraints. The Cys - Cys  $S_\gamma$  distances in these structures were examined in order to establish the probable bonding pattern. Prior to the calculations, the formation of disulphide bridges for 4 Cys residues (Cys12 - Cys28, Cys78 - Cys92) was already established with high probability by the observation of  $H_\beta$ - $H_\beta$  NOEs between these pairs of Cys residues. Examination of the initial structures confirmed these disulphide bridges and also indicated a disulphide bridge between residues Cys30 and Cys41. The third disulphide bridge in domain-1 (Cys7 - Cys18) could thus be assigned by default, although the structure of the N-terminus was not well-defined by the NMR data. The best six structures in terms of total X-PLOR energy and violations indicated that the average Cys - Cys  $S_\gamma$  distance was lowest for the disulphide bonding pattern [1-3, 2-4, 5-6] in each domain, and only this combination allowed all Cys residues to form contacts with a partner  $<3.5$  Å away. Thus, this disulphide bonding pattern was most consistent with the experimental data for both domains, and was imposed (initially as NOE-style distance restraints) in subsequent calculations. The [1-3, 2-4, 5-6] pattern is that expected for an EGF-like domain.

*Hydrogen Bonds:* Non-exchanging amide groups involved in stable hydrogen bonds were identified in spectra of samples examined in 100 %  $D_2O$ . The corresponding hydrogen bond acceptors were determined by examining the initial structural ensemble, using the Insight II and HBPlus (McDonald *et al.*, 1994) programs, and hydrogen bond distance restraints were included in subsequent calculations. Further hydrogen bonds were identified in a similar manner in iterative calculations. Only 10 backbone hydrogen bonds in the antiparallel  $\beta$  sheets were used as restraints. Two distance restraints were used for each hydrogen bond, 1.7 - 2.3 Å from proton to acceptor, and 3.0 - 3.6 Å from donor nitrogen atom to acceptor.

### Structure Calculations

All the structure calculations were performed following standard protocols for *ab initio* simulated annealing from an extended chain using X-PLOR version 3.843 on a Silicon Graphics Origin 200 computer. The initial calculations used an initial temperature of 1000K, and 9000 steps of 5 fs in the restrained molecular dynamics stage. A soft-square potential was used for distance restraints. The SHAKE (Ryckaert *et al.*, 1977) algorithm was employed during molecular dynamics to maintain correct bond lengths. Refinement used a square well potential for restraints, and a final slow cooling of 30000 steps of 4 fs each from 2000K. A modified "parallhdg.pro" force-field parameter set was used, with modifications to parameters for Arg and Pro residues, and for hydrogen bonds (Polshakov *et al.*, 1997). Force constants were 50 kcal mol<sup>-1</sup> Å<sup>-2</sup> for all distance restraints including hydrogen bonds, and 200 kcal mol<sup>-1</sup> rad<sup>-2</sup> for dihedral restraints. The N-terminal sequence including the vector-encoded residues and (His)<sub>6</sub> tag was excluded from the structure calculations. All peptide bonds were constrained to be trans. NOE data for all 5 Pro residues showed strong H<sub>α(i-1)</sub>-ProH<sub>α</sub> crosspeaks, consistent with the trans peptide conformation.

Initial structures were calculated as described above to determine the disulphide bonding pattern. Then the calculation was repeated with identical NOE-derived distance and dihedral angle restraints, with the addition of 6 distance restraints (1.92-3.12 Å) representing the disulphide bridges. A new set of 50 structures was obtained, from which the best 20 structures were selected. The criteria used for selection were that the structures were below the median value of both total X-PLOR energy and rms NOE difference, and had no dihedral angle violations. The resulting structures had good geometry and between zero and two NOE violations > 0.5 Å. These structures were used to assign previously ambiguous NOEs and to determine the hydrogen bonds as described above.

The final structure calculation and refinement used an expanded restraint list including hydrogen bonds, additional dihedral restraints, stereoassignments of β-methylene and Leu δ signals, and more precisely calibrated ROE data (see Table 1). A set of 100 structures

was obtained using this list, and 38 structures with 0-2 NOE violations  $> 0.5 \text{ \AA}$  and no dihedral angle violations  $> 5^\circ$  were accepted. These 38 structures were refined by the slow-cooling procedure described above, producing a final ensemble of 32 accepted structures with no NOE violations  $> 0.5 \text{ \AA}$  and no dihedral angle violations  $> 5^\circ$ . These selection criteria produced an ensemble of structures that extend to the end of the continuum of total potential energies in order to include structures having large scale correlated motions (Abseher *et al.*, 1998). Statistics for the final ensemble are given in Table 1. Coordinates for the 32 refined structures have been deposited in the Brookhaven Protein Data Bank (coordinates ID code 1cej; NMR restraints ID code r1cejmr).

Structures were analyzed during the calculation process using X-PLOR 3.8 (Nilges *et al.*, 1991), PROCHECK-NMR/AQUA (Laskowski *et al.*, 1996), and Insight II for quality of agreement with experimental data, precision, geometry, and energy. Models were aligned with Insight II and fitpdb, and displayed with Insight II, MOLSCRIPT (Kraulis, 1991), and GRASP (Nicholls *et al.*, 1991).

Table 1.

## A: RESTRAINTS SUMMARY

Number of conformers calculated: 100      Number of conformers accepted: 32

**Acceptance criteria:**No distance violation:  $> 0.5 \text{ \AA}$ No dihedral angle violation:  $> 5^\circ$ 25 **NOE/ROE distance restraints:**

Intraresidue: 73

Sequential: 222

Medium range ( 2-4 ): 90

Long range (  $> 4$  ): 185

Total: 570

30 **Dihedral angle restraints:**

phi: 25

psi: 33

chi-1: 22

chi-2: 5

Total: 85

Hydrogen bonds: 10 Disulphide bonds: 6

## B: STRUCTURE QUALITY

	<i>average</i>	<i>+/- s.d.</i>
5 <i>Total X-PLOR energy (kcal mol<sup>-1</sup>)</i>	168	20
<i>NOE X-PLOR energy (kcal mol<sup>-1</sup>)</i>	21	8
<i>rmsd NOE</i>	0.026	0.005
<i>rmsd dihedral angle</i>	0.236	0.095
10 <i>rmsd bond length</i>	0.0029	0.0002
<i>rmsd bond angle</i>	0.357	0.023
<i>rmsd improper</i>	0.266	0.018

### Backbone rmsd of structured region: (69 residues)

15 Overall:	1.05	0.28
Domain-1:	0.81	0.32
Domain-2:	0.83	0.35

### Ramachandran plot quality (phi/psi angles):

20 Most favoured	49.5 %
Additional allowed	42.1 %
Generously allowed	5.6 %
Disallowed	2.7 %

### 25 Monoclonal antibodies (mAbs)

Anti-MSP-1<sub>19</sub> monoclonal antibodies used in this study were : mouse IgG mAbs 1E1, 1E8, 2F10, 111.2, 111.4 2.2, 5.2, 7.5, 9C8, 12.8, 12.10, 12D11, 117.2, 8A12 (Holder *et al.*, 1985; McBride & Heidrich, 1987; Blackman *et al.*, 1987; Guevara Patiño *et al.*, 1997);  
 30 and mouse IgM mAb 5B1 (Pirson & Perkins, 1985). Of these, mAbs 12.8, 12.10 and 5B1 are neutralising, inhibitory antibodies and 1E1, 2.2, 7.5, 9C8 and 111.4 are blocking antibodies. Some antibodies such as 111.2 are neither inhibitory nor blocking.



### Construction of modified MSP-1<sub>19</sub> clones

The DNA coding the wild type MSP-1<sub>19</sub> domain of *Plasmodium falciparum* (T9-94/Wellcome strain) MSP-1 has been cloned in expression vector pGEX-3X to produce MSP-1<sub>19</sub> fused to the carboxy-terminus of the *Schistosoma japonicum* glutathione S-transferase (GST) in *Escherichia coli* (Burghaus & Holder, 1994). Site-directed mutagenesis of MSP-1<sub>19</sub> DNA sequence was done in either of two ways.

The first method was a modification of the method of Perrin & Gilliland (1990) to carry out polymerase chain reaction (PCR)-mediated site specific mutagenesis. DNA was amplified using the plasmid as a template together with one oligonucleotide to introduce the point mutation and a 5' primer from outside of the MSP-1<sub>19</sub> sequence. The amplified product was purified after electrophoresis on an agarose gel and used in a second amplification step together with a 3' primer from outside of the other end of the MSP-1<sub>19</sub> sequence and the plasmid as template. This second PCR product was digested with the restriction enzymes *Eco*R1 and *Bam*H1 and the product consisting of the modified MSP-1<sub>19</sub> coding sequence was inserted back into pGEX-3X and the products were used to transform DH5 $\alpha$  cells.

The second method used the QuikChange™ Site-directed mutagenesis kit from Stratagene. Briefly, using the plasmid pGEX-MSP-1<sub>19</sub> as a template, two complementary synthetic oligonucleotide primers containing the desired point mutation were designed and were extended on the template by temperature cycling with the enzyme *Pfu* DNA Polymerase. This incorporation of the oligonucleotide primers results in the generation of a mutated plasmid containing staggered nicks in the DNA sequence. Following the temperature cycling, the product was treated with *Dpn*I endonuclease which will digest the methylated parental DNA template and leaves the mutation-containing newly synthesised DNA intact. The DNA incorporating the desired mutation was then transformed into *E. coli* strain DH5 $\alpha$  (Life technologies) competent cells where the nicks will be repaired.

Clones were screened by analysis of restriction enzyme digests and by PCR screening of

the insert gene. The DNA sequence of the selected mutant clones was confirmed using a PerkinElmer Applied Biosystems ABI 377 automatic sequencer according to the manufacturer's instructions.

5    Expression of the GST-MSP-1<sub>19</sub> fusion proteins

Expression of GST-MSP-1<sub>19</sub> was induced with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG; Melford Laboratories) for 1 hour in the *E. coli* strain TOPP 1 (Stratagene). The cells were then harvested by centrifugation and the cell pellet was  
10    resuspended in cell lysis buffer (50 mM Tris-HCl/1 mM EDTA pH 8.0 containing 0.2% (v/v) Nonidet P40 (NP40; BDH). Phenylmethylsulphonyl fluoride (PMSF; Sigma) in isopropanol was added to a final concentration of 1 mM. The cell suspension was sonicated, on ice, using VibraCell sonicator (Sonics & Materials) at 50% duty cycle for 3 min (six 30 sec pulses with 30 sec in between). The cell lysate was centrifuged at 65000 x  
15    g for 1 hour at 4°C. Supernatant containing soluble GST-fusion protein was applied to a glutathione-agarose column (Sigma) and the GST-fusion protein was eluted with 5 mM reduced glutathione. The eluted GST-fusion protein was dialysed extensively against phosphate buffered saline (PBS) at 4°C.

20    SDS-PAGE and Western Blotting

Proteins were analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). Samples were solubilised in SDS-PAGE buffer without reducing agents, then fractionated on a homogeneous 12.5% polyacrylamide gel. The pre-  
25    stained low range molecular mass markers (24-102kDa) from Bio-Rad were used as markers. When required, SDS-PAGE-fractionated polypeptides were either stained with Coomassie Brilliant Blue R-250 (CBB; Sigma) or electrophoretically transferred to Optitran BA-S 83 reinforced nitrocellulose (Schleicher & Schull, 0.2  $\mu$ m pore size) for analysis by western blotting. Blots were blocked with 5% BSA, 0.5% Tween 20 in PBS  
30    (PBS-T) for 1 h at room temperature, then washed in PBS-T. Blots were probed with first antibodies for 2 h at room temperature, washed 3 times in PBS-T, and then incubated in 1/1000 dilution of horse radish peroxidase (HRP)-conjugated sheep anti-mouse IgG (H+L)

(ICN Immunobiologicals) or Goat anti-mouse IgM ( $\mu$  chain) (Sigma) for 1 h at room temperature. Blots were then washed 3 times in PBS-T and developed using Super Signal Substrate (Pierce) as HRP substrate for 1 min. Blots were then placed in plastic wrap and exposed to X-ray film (XB-200, X-ograph Imaging Systems). The films were processed  
5 with an Agfa Gevamat60 film processor (Agfa).

#### Analysis of antibody-antigen interaction using a BIAcore machine

GST-MSP-1<sub>19</sub> containing either the wild type or various modified sequences was used to  
10 coat a carboxymethyl dextran hydrogen sensor chip by the following methodology. The binding of the GST-MSP-1<sub>19</sub> was via amino groups using EDC/NHS chemistry. Immobilisation was done with the amine coupling kit (Pharmacia BIAcore). The CM dextran surface was activated with 50  $\mu$ l of 200 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 5 mM N-hydroxysuccinamide (NHS) for 10 min. GST-MSP-1<sub>19</sub>  
15 was then coupled to the BIAcore sensor surface using 50  $\mu$ l of a solution at 100  $\mu$ g ml<sup>-1</sup> in coating buffer (0.01M sodium acetate buffer, pH 3.5) for 10 min. Unreacted carboxyl groups were blocked by adding 50  $\mu$ l 1 M ethanolamine, pH 8.5 for 10 min. The cells were washed with two pulses of 20  $\mu$ l 10 mM glycine-HCl, pH 2.8 for 8 min in total to remove any non-covalently bound protein. The immobilisation procedure was carried out  
20 at a flow rate of 5  $\mu$ l min<sup>-1</sup>. Measurements were performed on the BIAcore 2000 instrument.

## RESULTS

### **Example 1 - Resonance assignments, NMR restraints and structure determination**

  
25

The assignments and restraints were obtained as described in Materials and Methods using a range of multidimensional heteronuclear experiments with <sup>13</sup>C/<sup>15</sup>N uniformly labelled protein. Sample spectra from 3D and 4D experiments showing NOE connections to the  
30 Lys35 backbone amide NH proton, resolved and unambiguously assigned using the <sup>13</sup>C chemical shift information, are shown in Figure 2. The distance, dihedral angle and hydrogen bond restraints used in the final set of structure calculations are summarized in

Table 1. A total of 570 unambiguously assigned distance restraints, 85 dihedral angle restraints, and 10 hydrogen bonds were used in the final set. The assignments and restraint list shown in Table A have been submitted to the BioMagResBank database. Three disulphide bonds, with the (1-3, 2-4, 5-6) pattern for each domain were experimentally  
5 determined from the NMR data in preliminary calculations as described in Materials and Methods, and these were also included in the final refinement. A final set of 32 models was calculated and refined using these restraints and these structures are shown in Figure 3 superimposed on the backbone of the representative structure  $S_{rep}$ . Table 1 shows that all 32 models have good geometry and are in good agreement with the experimental data  
10 with no NOE violations  $> 0.5 \text{ \AA}$  and no dihedral angle violations  $> 5^\circ$ . The atomic rmsd value for the backbone atoms of the well-structured region (residues 15 - 64, 74 - 92) is  $1.05 \text{ \AA}$  (see Table 1). The local backbone rmsd is highest at the N-terminus (up to Cys12), in the loop Glu65 - Lys73, and following Cys92 at the C-terminus. The Ramachandran plot quality is typical of that found for other EGF structures (Doreleijers *et al.*, 1998).

## Description of the structure

### *EGF-domains*

Analysis of the final ensemble by PROCHECK-NMR indicated that each domain contains  
20 a major stretch of antiparallel  $\beta$ -sheet containing the third and fourth Cys residues of each domain, as expected for an EGF-like fold, as well as an additional minor antiparallel  $\beta$ -sheet at the C-terminal end of domain-1, similar to some (but not all) EGF family members. These secondary structure features, together with the disulphide bonding patterns, can be seen in Figure 4. There is also a well-defined type II tight turn in domain-  
25 1, with a hydrogen bond from Tyr 34NH proton to Leu31 carbonyl oxygen. The normally conserved EGF consensus Gly residue in the tight turn is replaced in domain-1 by a residue with a positive  $\phi$  angle (Asn33), while the conserved aromatic residue is present (Tyr34). There is a probable hydrogen bond between Leu 31NH proton to Asn15 carbonyl oxygen. Domain-2 contains two turns preceding the major  $\beta$ -sheet, (Asn53 - Cys56,  
30 Asp57 - Ala60), and a final bend from Leu86 - Phe91 with a probable hydrogen bond from Asp57 NH proton to the carbonyl oxygen of Ile90 or Gly89. A surface-exposed loop from Pro81 to Pro85 replaces the tight turn, while the aromatic residue is not conserved.

The large loop at the end of the major  $\beta$ -sheet (Glu65 - Lys73) is relatively disordered, and high mobility for the segment Gly68 - Gly71 was confirmed by backbone amide  $^{15}\text{N}\{^1\text{H}\}$  heteronuclear NOE measurements (Barbato *et al.*, 1992). The heteronuclear NOE values are dramatically reduced for residues in this region. At the N-terminus: the low NOE intensities correspond to increased mobility compared with the rest of the protein. The interdomain linker region from Pro45 to Pro47 is distinct from other EGF-like module pairs. The conformations of the disulphide bridges between Cys30 - Cys41 in domain-1, and the three Cys - Cys bonds in domain-2 are all left handed spirals (Richardson, 1981). Bridges between Cys30 - Cys41, Cys56 - Cys76, and Cys78 - Cys92 are particularly close to their equivalents in the blood coagulation factor Xa structure (1hcg). The conformations of the first two disulphide bridges in the relatively disordered N-terminal segment of domain-1 were not determined.

Figure 5 shows the backbone C, N,  $\text{C}_\alpha$  atom alignments of the two MSP-1 C-terminal fragment domains made with typical examples of EGF-like domains from several proteins, using the fitpdb program. Pairwise alignments showed that the two domains from MSP-1 are more similar to the factor Xa structure and its close relative from Clr, than to each other or to the other structures tested. The rmsd values for MSP-1 domains compared to factor Xa are comparable to those of the more distantly related structures fibrillin-1 and transforming growth factor- $\alpha$ .

The overall fold of each MSP-1 domain is thus similar to typical EGF family members, with the turns following the fifth Cys residue roughly equivalent, in spite of the divergence from the EGF consensus (C(5)xxG $\alpha$ ) where  $\alpha$  is a Phe or Tyr residue. Although some of the external loops are disordered, the scaffold is quite stable, as indicated by the non-exchangeable backbone amides (see above and in Protein Data Bank/BioMagResBank submission for details).

Unlike many EGF-like domains such as fibrillin-1, the MSP-1 C-terminal fragment lacks the conserved EGF  $\text{Ca}^{2+}$ -binding sequence and there was no evidence of  $\text{Ca}^{2+}$  binding to the MSP-1 C-terminal fragment. The 2D  $^1\text{H}$ -NOESY spectra were virtually identical in the absence or presence of 20 mM  $\text{CaCl}_2$ , indicating that any binding that might occur has,

at most, only a small affect the overall structure.

### *Domain interface and surface*

The most striking feature of the MSP-1 C-terminal fragment structure is the interface  
5 between the domains, which consists of several nonpolar amino acids (Phe19, Leu31,  
Leu32, Leu86, Phe87, Ile90 and Phe91) involved in hydrophobic interactions. These  
residues join the base of the major  $\beta$ -sheet and the tight turn in domain-1 with the final  
bend from residue 86 to 91 in domain-2. The domain interactions result in the domains  
forming a U-shaped structure which contrasts with structures observed for other pairs of  
10 EGF domains (Downing *et al.*, 1996; Brandstetter *et al.*, 1995). For example, in fibrillin-1,  
the interface between EGF domains 32 and 33 is largely formed by a shared  $\text{Ca}^{2+}$  ligation  
site (Downing *et al.*, 1996), and the overall structure resembles a rigid rod, with distant N-  
and C-termini. This contrasts with MSP-1 where the EGF-like domains are folded against  
each other so that their termini are relatively close together. A comparison of fibrillin-1  
15 and MSP-1 EGF module pairs is shown in Figure 6. Although both termini of the MSP-1  
C-terminal fragment are somewhat disordered, NOE contacts were observed between  
nuclei in the two ends. The proximity of the C- and N-terminal positions may be  
significant, since it suggests that the proteolytic processing site that produces the C-  
terminal 96 amino acid fragment may be very close to the GPI membrane attachment site  
20 at or near residue 96. This proximity is consistent with the idea that a membrane-bound  
*Plasmodium* proteinase is responsible for secondary processing.

The electrostatic potential surface of the MSP-1 C-terminal fragment is shown in two  
views in Figure 7. The surface in Figure 7a is highly charged, especially in the protruding  
25 loop regions 23 - 27, 35 - 40 and 64 - 66. The surface in Figure 7b contains more neutral  
hydrophilic residues as well as a small hydrophobic patch from Pro85 - Phe87 near the  
center of the surface. In the future, such information could assist in understanding how  
these different surfaces may be involved in interactions with the rest of the MSP-1  
precursor, the processing proteinase, other proteins on the merozoite surface, or unknown  
30 targets on the erythrocyte or parasite vacuolar membrane surfaces.

### Primary sequence conservation

The residues involved in the hydrophobic domain interface in *P. falciparum* are also shown in Figure 1, together with corresponding residues in MSP-1 of the less virulent human malaria parasite, *P. vivax* (Del Portillo *et al.*, 1991; Gibson *et al.*, 1992). Extensive conservation of the interface residues (with conservative substitutions) suggests that *P. vivax* and perhaps other *Plasmodium* species as well, may have a similar U-shaped EGF module pair arrangement. Another feature of the *P. vivax* sequence, also seen in other *Plasmodium* species, is the single disulphide bond deficiency in the first EGF-like domain resulting from the absence of cysteine residues equivalent to the *P. falciparum* Cys12 and Cys28.

### *P. falciparum* dimorphic sites

Five dimorphic sites have been observed in the *P. falciparum* MSP-1<sub>19</sub> C-terminal fragment from different isolates (Qari *et al.*, 1998). Several observations can be made about the position of these sites on the MSP-1 structure. Two sites, Gln14/Glu14 and Lys61/Thr61, involve residues in relatively well-structured backbone regions, with surface-exposed hydrophilic or charged side-chains. A pair of adjacent sites, with the sequence variants Asn70 - Gly71/Ser70 - Arg71, occurs in the disordered loop of domain-2, within a segment (residues 68 - 71) that has been shown to be highly mobile. The region from Glu65 to Lys73 also appears to be the most variable region among different *Plasmodium* species (Daly *et al.*, 1992; Holder *et al.*, 1992). Finally, the fifth site has a substitution between hydrophobic residues (Leu86/Phe86). This partially-exposed side-chain is located at the hydrophobic domain interface, and the conservative substitution is consistent with a role in this interaction.

### Example 2 - Mutation and Monoclonal Antibody Binding studies

As a step towards understanding antibody interactions with the MSP-1 C-terminal fragment, the effect of engineered point mutations (within domain-1) on antibody binding has been studied. Amino acid substitutions were made that consisted of radical changes.

These radical changes consisted of, for example, replacing an aliphatic residue with a charged polar residue, replacing a positively charged side chain with a negatively charged side chain, replacing an amino acid with a large side chain with an amino acid with a smaller or no side chain (glycine), replacing a polar amino acid with a charged polar amino acid, replacing a polar amino acid with an aromatic amino acid, replacing a large aromatic amino acid with an amino acid with a small side chain, and replacing cysteine residues that are involved in disulphide bonds.

Four individual amino acid substitutions shown in Figure 8, each completely abolish binding of one or more mAbs to the mutant fragment, as detected by Western blotting. The Glu26 mutation, shown in cyan, is closest to the N-terminal proteolytic processing site (magenta) at Asn1, and is the only one of this group of mutations that affects binding of a processing-inhibitory antibody, i.e. one that is capable of preventing both proteolytic processing of the MSP-1 precursor and erythrocyte invasion *in vitro*. The other three mutations abolish binding of blocking antibodies that bind to the native C-terminal fragment and interfere with the binding of processing-inhibitory antibodies.

Additional mutations were made based on the immunochemical analyses and the tertiary structure of the molecule, and the binding of the mAbs was assessed by western blotting and BIAcore analysis. The results are summarised in Table 2. The results of the binding of selected mAbs to the modified proteins as detected by Western blotting are shown in Figure 9, and by BIAcore analysis in Figure 10. Some individual amino acid changes have no effect on the binding of any of the mAbs tested (for example Leu22 to Arg). Other substitutions affect the binding of one or more mAbs.

Of particular interest are those changes that prevent the binding of blocking antibodies but have no effect on the binding of the inhibitory antibodies. For example, replacement of Asn15 by Arg prevents the binding of mAb 7.5, replacement of Glu27 by Tyr prevents the binding of mAb 2.2, replacement of Leu31 by Arg prevents the binding of mAb 1E1, replacement of Tyr34 by Ser prevents the binding of mAb 7.5, and replacement of Glu43 by Leu prevents the binding of mAb 111.4.



Several combinations of substitutions that prevent the binding of blocking antibodies but do not affect the binding of inhibitory antibodies were made in single proteins (Table 2 and Figure 11). In the first Glu27→Tyr, Leu31→Arg and Glu43→Leu were combined, in the second Glu27→Tyr, Leu31→Arg, Tyr34→Ser, and Glu43→Leu were combined, and the third Asn15→Arg, Glu27→Tyr, Leu31→Arg and Glu43→Leu were combined. None of these modified proteins bound any of the blocking antibodies but continued to bind the inhibitory antibodies. We propose that the mutant proteins will induce a polyclonal response that is more inhibitory than that induced by the wild type protein.

The modified recombinant proteins will also be used to affinity select antibodies from pooled serum from individuals exposed to malaria. We hypothesise that the modified proteins will select less blocking antibody than the wild type protein and that therefore these selected antibodies will be more effective in inhibiting parasite invasion *in vitro* and secondary processing.

In the first EGF-like domain of MSP-1 from the rodent, primate and *P. vivax* malaria parasites, cysteines 2 and 4 are not present. We have replaced this cysteine pair (Cys12 and Cys28) in the *P. falciparum* protein. This does not appear to have any effect on the binding of any of the inhibitory antibodies, but does abolish the binding of the blocking antibody mAb 2.2. We propose that one reason why the proteins from these other malaria parasites are more immunogenic is that T cell recognition is more effective or that processing by antigen processing cells proceeds by a different degradation pathway that drives the fine specificity of the antibody response in a more productive direction (see for example Egan *et al.*, 1997). Removal of the cysteine pair may improve the immunogenicity of the modified protein and this will be assessed by measuring the level of antibodies induced by the *P. falciparum* protein without the two cysteines with the level of antibodies induced by the wild type protein.

46

Table 2 - The location of amino acid sequence changes and their effect on the binding of monoclonal antibodies

Position	Amino acid		Monoclonal Antibody binding														
	Wild type	mutant	12.8	12.10	5B1	1E1	2.2	7.5	111.4	111.2	9C8	2F10	12D11	117.2	5.2	1E8	8A12
6	Gln	Ile	++	++	++	++	+	+	++	++	++	++	++	++	++	++	++
14	Gln	Gly	++	++	++	++	++	+	++	++	++	++	++	+	++	++	++
14	Gln	Arg	++	++	++	++	++	+	++	++	+	++	++	++	++	++	++
15	Asn	Arg	++	++	++	++	++	+	++	++	++	++	+	++	++	+	++
20	Arg	Glu	+	++	+	++	++	++	++	++	++	++	++	++	++	+	++
22	Leu	Arg	++	++	++	++	++	++	++	++	+	++	++	++	++	+	++
24	Glu	Lys	+	++	++	++	++	++	++	++	++	++	++	++	++	+	++
25	Arg	Gly	++	++	++	++	++	++	++	++	++	++	++	++	++	+	+
26	Glu	Ile	-	++	+	++	-	++	++	++	++	++	++	++	++	++	+
27	Glu	Tyr	++	++	++	++	+	++	++	++	++	++	++	++	++	++	++
29	Lys	Ser	+	++	++	++	++	++	++	++	+	++	++	++	++	++	+
31	Leu	Arg	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++
32	Leu	Arg	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
33	Asn	Ile	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
34	Tyr	Ser	++	++	++	++	++	+	++	++	+	++	+	++	++	++	++
34	Tyr	Ile	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
35	Lys	Ile	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
36	Gln	Gly	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
37	Glu	Ile	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
39	Asp	Thr	++	++	++	++	++	++	++	++	++	++	++	++	++	+	++
40	Lys	Ile	+	++	+	+	++	++	+	++	+	++	+	+	++	++	+
43	Glu	Leu	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
48	Thr	Lys	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
53	Asn	Arg	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
80	Lys	Ile	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Wild type			++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

++ = strong binding, + = binding, - = no binding

-47-

Table 2 (cont.)

Position Combinations	Amino acid		Monoclonal antibody binding														
	Wildtype	Mutant	12.8	12.10	5B1	IE1	2.2	7.5	111.4	111.2	9C8	2F10	12D11	117.2	5.2	IE8	8A12
12+28	Cys	Ile	++	++	++	++	-	++	++	+	+	++	++	+	-	++	++
	Cys	Trp	++	++	++	++	-	++	++	++	++	++	++	++	-	++	++
12+28	Cys	Ala	++	++	++	++	-	++	++	++	++	++	++	++	-	-	-
	Cys	Phe	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-
14+18	Gln	Gly	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	Cys	Tyr	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
14+18	Gln	Arg	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	Cys	Tyr	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
34+39	Tyr	Ser	++	++	++	+	+	+	+	++	++	++	++	++	+	++	++
	Asp	Asn	++	++	++	+	+	+	-	-	+	-	+	+	++	++	+
43+48	Glu	Leu	++	++	++	+	+	+	-	-	+	+	+	+	++	++	+
	Thr	Ile	++	++	++	++	++	++	-	++	++	+	++	++	++	++	++
43+48	Glu	Leu	++	++	++	++	++	+	-	++	++	+	++	++	++	++	++
	Thr	Asn	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-
47+48	Pro	Ser	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-
	Thr	Lys	+	+	+	+	+	+	-	-	-	++	++	+	++	+	+
27+31+43	Glu	Tyr	++	++	++	-	-	+	-	-	-	++	++	+	++	+	++
	Leu	Arg	++	++	++	-	-	-	-	-	-	++	++	+	++	+	++
27+31+34+43	Glu	Leu	++	++	++	-	-	-	-	-	-	++	++	+	++	+	++
	Leu	Arg	++	++	++	-	-	-	-	-	-	++	++	+	++	+	++
15+27+31+43	Tyr	Ser	++	++	++	-	-	-	-	-	-	++	++	+	++	+	++
	Glu	Leu	++	++	++	-	-	-	-	-	-	++	++	+	++	+	++
15+27+31+43	Asn	Arg	++	++	++	-	-	-	-	-	-	++	++	+	++	+	++
	Glu	Tyr	++	++	++	-	-	-	-	-	-	++	++	+	++	+	++
15+27+31+43	Leu	Arg	++	++	++	-	-	-	-	-	-	++	++	+	++	+	++
	Glu	Leu	++	++	++	-	-	-	-	-	-	++	++	+	++	+	++
12+15+27+31+43	Cys	Ile	-	-	-	-	-	-	-	-	-	++	++	-	-	-	++
	Asn	Arg	-	-	-	-	-	-	-	-	-	++	++	-	-	-	++
12+15+27+31+43	Glu	Tyr	-	-	-	-	-	-	-	-	-	++	++	-	-	-	++
	Leu	Arg	-	-	-	-	-	-	-	-	-	++	++	-	-	-	++
12+15+27+31+43	Glu	Leu	-	-	-	-	-	-	-	-	-	++	++	-	-	-	++
	Glu	Leu	-	-	-	-	-	-	-	-	-	++	++	-	-	-	++

++ = strong binding, + = binding, - = no binding

TABLE A

```
#
# 13-10-98
# merozoite surface protein-1 (MSP-1) Plasmodium falciparum (C-terminal fragment)
# Reference:      1H : DSS=0.000    dioxane=3.755    (internal)
#                  15N: indirect
#                  13C: indirect
# 25C   pH 6.5   50mM NaPO4 100mM NaCl 90%H2O/10%D2O
# --FORMAT--
# BioMagResBank
#
# The original sequence entered was:
#
NISQHQCVKKQCPQNSGCFRHLDEREECKCLLNKQEGDKCVENPNPTCNENNGGCDADAKCTEEDSGSNGK
KITCECTKPDSYPLFDGIFCSSSN
#
# Expressed in NMR-STAR, this sequence is:
```

```
_Mol_residue_sequence
```

```
;
NISQHQCVKKQCPQNSGCFR
HLDEREECKCLLNKQEGDK
CVENPNPTCNENNGGCDADA
KCTEEDSGSNGKKITCECTK
PDSYPLFDGIFCSSSN
;
```

```
loop_
```

```
  _Residue_seq_code
  _Residue_author_seq_code
  _Residue_label
```

1 @ ASN	2 @ ILE	3 @ SER	4 @ GLN	5 @ HIS
6 @ GLN	7 @ CYS	8 @ VAL	9 @ LYS	10 @ LYS
11 @ GLN	12 @ CYS	13 @ PRO	14 @ GLN	15 @ ASN
16 @ SER	17 @ GLY	18 @ CYS	19 @ PHE	20 @ ARG
21 @ HIS	22 @ LEU	23 @ ASP	24 @ GLU	25 @ ARG
26 @ GLU	27 @ GLU	28 @ CYS	29 @ LYS	30 @ CYS
31 @ LEU	32 @ LEU	33 @ ASN	34 @ TYR	35 @ LYS
36 @ GLN	37 @ GLU	38 @ GLY	39 @ ASP	40 @ LYS
41 @ CYS	42 @ VAL	43 @ GLU	44 @ ASN	45 @ PRO
46 @ ASN	47 @ PRO	48 @ THR	49 @ CYS	50 @ ASN
51 @ GLU	52 @ ASN	53 @ ASN	54 @ GLY	55 @ GLY
56 @ CYS	57 @ ASP	58 @ ALA	59 @ ASP	60 @ ALA
61 @ LYS	62 @ CYS	63 @ THR	64 @ GLU	65 @ GLU
66 @ ASP	67 @ SER	68 @ GLY	69 @ SER	70 @ ASN
71 @ GLY	72 @ LYS	73 @ LYS	74 @ ILE	75 @ THR
76 @ CYS	77 @ GLU	78 @ CYS	79 @ THR	80 @ LYS
81 @ PRO	82 @ ASP	83 @ SER	84 @ TYR	85 @ PRO
86 @ LEU	87 @ PHE	88 @ ASP	89 @ GLY	90 @ ILE
91 @ PHE	92 @ CYS	93 @ SER	94 @ SER	95 @ SER
96 @ ASN				

```
stop_
```

```
#####
#   Chemical Shift Ambiguity Code Definitions   #
#                                               #
#   Codes   Definition                         #
#                                               #
#   1       Unique                           #
#   2       Ambiguity of geminal atoms or geminal methyl #
#           proton groups                     #
#   3       Aromatic atoms on opposite sides of the ring#
#           (e.g. Tyr HE1 and HE2 protons)   #
#   4       Intraresidue ambiguities (e.g. Lys HG and #
#           HD protons)                       #
#   5       Interresidue ambiguities (Lys 12 vs. Lys 27) #
#   9       Ambiguous, specific ambiguity not defined #
#                                               #
#####
```

# # INSTRUCTIONS

- # 1) Replace the @-signs with appropriate values.
- # 2) Text comments concerning the assignments can be supplied in the full deposition.
- # 3) Feel free to add or delete rows to the table as needed.
- # The row numbers (\_Atom\_shift\_assign\_ID values) will be re-assigned to sequential values by BMRB

# # The atom table chosen for this sequence is:

```
loop_
  _Atom_shift_assign_ID
  _Residue_seq_code
  _Residue_label
  _Atom_name
  _Atom_type
  _Chem_shift_value
  _Chem_shift_value_error
  _Chem_shift_ambiguity_code
```

#

Table A1. Supplementary: <sup>1</sup>H, <sup>13</sup>C and <sup>15</sup>N chemical shift assignments of MSP-1 C-terminal fragment

Atom shift assign	Residue Seq no.	Residue Name	Atom Name	Atom Type	Shift/ ppm	Error/ ppm	Ambiguity- Code
1	1	ASN	H	H	8.29	0.02	1
2	1	ASN	HA	H	4.60	0.02	1
3	1	ASN	HB2	H	2.86	0.02	2
4	1	ASN	HB3	H	2.75	0.02	2
5	1	ASN	HD21	H	?	?	?
6	1	ASN	HD22	H	?	?	?
7	1	ASN	C	C	?	?	?
8	1	ASN	CA	C	55.5	0.6	1
9	1	ASN	CB	C	40.9	0.6	1
11	1	ASN	N	N	125.8	0.3	1
12	1	ASN	ND2	N	?	?	?
13	2	ILE	H	H	8.29	0.02	1

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14	2	ILE	HA	H	4.25	0.02	1
15	2	ILE	HB	H	1.97	0.02	1
16	2	ILE	HG12	H	1.39	0.02	2
17	2	ILE	HG13	H	1.19	0.02	2
18	2	ILE	HG2	H	0.92	0.02	1
19	2	ILE	HD1	H	0.81	0.02	1
20	2	ILE	C	C	173.8	0.6	1
21	2	ILE	CA	C	62.2	0.6	1
22	2	ILE	CB	C	38.7	0.6	1
23	2	ILE	CG1	C	27.5	0.6	1
24	2	ILE	CG2	C	18.2	0.6	1
25	2	ILE	CD1	C	13.7	0.6	1
26	2	ILE	N	N	121.1	0.3	1
27	3	SER	H	H	8.47	0.02	1
28	3	SER	HA	H	4.20	0.02	1
29	3	SER	HB2	H	3.90	0.02	1
30	3	SER	HB3	H	3.90	0.02	1
32	3	SER	C	C	?	?	?
33	3	SER	CA	C	60.9	0.6	1
34	3	SER	CB	C	63.3	0.6	1
35	3	SER	N	N	119.3	0.3	1
36	4	GLN	H	H	8.32	0.02	1
37	4	GLN	HA	H	4.02	0.02	1
38	4	GLN	HB2	H	1.88	0.02	1
39	4	GLN	HB3	H	1.88	0.02	1
40	4	GLN	HG2	H	1.75	0.02	1
41	4	GLN	HG3	H	1.75	0.02	1
42	4	GLN	HE21	H	?	?	?
43	4	GLN	HE22	H	?	?	?
44	4	GLN	C	C	?	?	?
45	4	GLN	CA	C	57.7	0.6	1
46	4	GLN	CB	C	27.9	0.6	1
47	4	GLN	CG	C	32.7	0.6	1
49	4	GLN	N	N	121.6	0.3	1
50	4	GLN	NE2	N	?	?	?
51	5	HIS	H	H	7.76	0.02	9
52	5	HIS	HA	H	5.09	0.02	1
53	5	HIS	HB2	H	2.70	0.02	1
54	5	HIS	HB3	H	2.70	0.02	1
56	5	HIS	HD2	H	6.87	0.02	1
57	5	HIS	HE1	H	7.92	0.02	1
59	5	HIS	C	C	175.7	0.6	1
60	5	HIS	CA	C	54.8	0.6	1
61	5	HIS	CB	C	29.2	0.6	1
65	5	HIS	N	N	113.6	0.3	9
68	6	GLN	H	H	7.42	0.02	9
69	6	GLN	HA	H	4.43	0.02	1
70	6	GLN	HB2	H	2.05	0.02	1
71	6	GLN	HB3	H	2.05	0.02	1
72	6	GLN	HG2	H	2.42	0.02	1
73	6	GLN	HG3	H	2.42	0.02	1
74	6	GLN	HE21	H	7.59	0.02	5
75	6	GLN	HE22	H	6.92	0.02	5
76	6	GLN	C	C	175.7	0.6	1
77	6	GLN	CA	C	55.1	0.6	1
78	6	GLN	CB	C	28.8	0.6	1
79	6	GLN	CG	C	33.8	0.6	1
81	6	GLN	N	N	122.5	0.3	9

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82	6	GLN	NE2	N	112.6	0.3	5
83	7	CYS	H	H	9.18	0.02	1
84	7	CYS	HA	H	4.09	0.02	1
85	7	CYS	HB2	H	3.31	0.02	2
86	7	CYS	HB3	H	3.11	0.02	2
88	7	CYS	C	C	174.4	0.6	1
89	7	CYS	CA	C	56.6	0.6	1
90	7	CYS	CB	C	42.3	0.6	1
91	7	CYS	N	N	124.5	0.3	1
92	8	VAL	H	H	10.42	0.02	1
93	8	VAL	HA	H	4.33	0.02	1
94	8	VAL	HB	H	2.15	0.02	1
95	8	VAL	HG1	H	0.84	0.02	2
96	8	VAL	HG2	H	0.82	0.02	2
97	8	VAL	C	C	176.6	0.6	1
98	8	VAL	CA	C	62.5	0.6	1
99	8	VAL	CB	C	34.4	0.6	1
100	8	VAL	CG1	C	21.5	0.6	2
101	8	VAL	CG2	C	19.7	0.6	2
102	8	VAL	N	N	119.1	0.3	1
103	9	LYS	H	H	9.42	0.02	1
104	9	LYS	HA	H	4.51	0.02	1
105	9	LYS	HB2	H	1.81	0.02	4
106	9	LYS	HB3	H	1.81	0.02	4
107	9	LYS	HG2	H	1.41	0.02	4
108	9	LYS	HG3	H	1.41	0.02	4
109	9	LYS	HD2	H	?	?	?
110	9	LYS	HD3	H	?	?	?
111	9	LYS	HE2	H	3.33	0.02	1
112	9	LYS	HE3	H	3.33	0.02	1
114	9	LYS	C	C	?	?	?
115	9	LYS	CA	C	57.7	0.6	1
116	9	LYS	CB	C	?	?	?
117	9	LYS	CG	C	?	?	?
118	9	LYS	CD	C	?	?	?
119	9	LYS	CE	C	?	?	?
120	9	LYS	N	N	124.1	0.03	1
122	10	LYS	H	H	8.94	0.02	1
123	10	LYS	HA	H	4.06	0.02	1
124	10	LYS	HB2	H	1.86	0.02	1
125	10	LYS	HB3	H	1.86	0.02	1
126	10	LYS	HG2	H	1.29	0.02	1
127	10	LYS	HG3	H	1.29	0.02	1
128	10	LYS	HD2	H	1.70	0.02	2
129	10	LYS	HD3	H	1.59	0.02	2
130	10	LYS	HE2	H	3.04	0.02	1
131	10	LYS	HE3	H	3.04	0.02	1
133	10	LYS	C	C	?	?	?
134	10	LYS	CA	C	57.1	0.6	1
135	10	LYS	CB	C	34.3	0.6	1
136	10	LYS	CG	C	25.6	0.6	1
137	10	LYS	CD	C	29.6	0.6	1
138	10	LYS	CE	C	42.4	0.6	1
139	10	LYS	N	N	122.4	0.3	1
141	11	GLN	H	H	?	?	?
142	11	GLN	HA	H	4.47	0.02	1
143	11	GLN	HB2	H	2.03	0.02	2
144	11	GLN	HB3	H	1.89	0.02	2

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145	11	GLN	HG2	H	2.28	0.02	1
146	11	GLN	HG3	H	2.28	0.02	1
147	11	GLN	HE21	H	7.45	0.02	2
148	11	GLN	HE22	H	6.84	0.02	2
149	11	GLN	C	C	?	?	?
150	11	GLN	CA	C	54.4	0.6	1
151	11	GLN	CB	C	28.7	0.6	1
152	11	GLN	CG	C	33.8	0.6	1
154	11	GLN	N	N	?	?	?
155	11	GLN	NE2	N	112.9	0.3	1
156	12	CYS	H	H	?	?	?
157	12	CYS	HA	H	5.09	0.02	1
158	12	CYS	HB2	H	3.49	0.02	2
159	12	CYS	HB3	H	2.34	0.02	2
161	12	CYS	C	C	?	?	?
162	12	CYS	CA	C	52.4	0.6	1
163	12	CYS	CB	C	37.2	0.6	1
164	12	CYS	N	N	?	?	?
165	13	PRO	HA	H	4.55	0.02	1
166	13	PRO	HB2	H	2.45	0.02	1
167	13	PRO	HB3	H	1.94	0.02	1
168	13	PRO	HG2	H	1.73	0.02	2
169	13	PRO	HG3	H	2.04	0.02	2
170	13	PRO	HD2	H	3.43	0.02	2
171	13	PRO	HD3	H	3.80	0.02	2
172	13	PRO	C	C	176.4	0.6	1
173	13	PRO	CA	C	62.6	0.6	1
174	13	PRO	CB	C	33.0	0.6	1
175	13	PRO	CG	C	27.5	0.6	1
176	13	PRO	CD	C	50.6	0.6	1
178	14	GLN	H	H	8.48	0.02	1
179	14	GLN	HA	H	4.01	0.02	1
180	14	GLN	HB2	H	1.94	0.02	1
181	14	GLN	HB3	H	1.94	0.02	1
182	14	GLN	HG2	H	2.42	0.02	1
183	14	GLN	HG3	H	2.42	0.02	1
184	14	GLN	HE21	H	7.59	0.02	5
185	14	GLN	HE22	H	6.92	0.02	5
186	14	GLN	C	C	176.6	0.6	1
187	14	GLN	CA	C	57.4	0.6	1
188	14	GLN	CB	C	28.6	0.6	1
189	14	GLN	CG	C	33.8	0.6	1
191	14	GLN	N	N	120.5	0.3	1
192	14	GLN	NE2	N	112.6	0.3	5
193	15	ASN	H	H	8.93	0.02	1
194	15	ASN	HA	H	3.77	0.02	1
195	15	ASN	HB2	H	2.58	0.02	1
196	15	ASN	HB3	H	1.09	0.02	1
197	15	ASN	HD21	H	6.97	0.02	1
198	15	ASN	HD22	H	7.12	0.02	1
199	15	ASN	C	C	171.9	0.6	1
200	15	ASN	CA	C	54.6	0.6	1
201	15	ASN	CB	C	36.3	0.6	1
203	15	ASN	N	N	115.8	0.3	1
204	15	ASN	ND2	N	115.4	0.3	1
205	16	SER	H	H	7.34	0.02	1
206	16	SER	HA	H	4.93	0.02	1
207	16	SER	HB2	H	3.62	0.02	2



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208	16	SER	HB3	H	3.52	0.02	2
210	16	SER	C	C	173.5	0.6	1
211	16	SER	CA	C	57.5	0.6	1
212	16	SER	CB	C	67.9	0.6	1
213	16	SER	N	N	109.9	0.3	1
214	17	GLY	H	H	8.92	0.02	1
215	17	GLY	HA2	H	3.83	0.02	1
216	17	GLY	HA3	H	2.06	0.02	1
217	17	GLY	C	C	?	?	?
218	17	GLY	CA	C	42.6	0.6	1
219	17	GLY	N	N	108.5	0.3	1
220	18	CYS	H	H	7.01	0.02	1
221	18	CYS	HA	H	5.63	0.02	1
222	18	CYS	HB2	H	3.01	0.02	1
223	18	CYS	HB3	H	3.01	0.02	1
225	18	CYS	C	C	172.5	0.6	1
226	18	CYS	CA	C	55.8	0.6	1
227	18	CYS	CB	C	43.2	0.6	1
228	18	CYS	N	N	120.5	0.3	1
229	19	PHE	H	H	9.12	0.02	1
230	19	PHE	HA	H	4.43	0.02	1
231	19	PHE	HB2	H	1.70	0.02	2
232	19	PHE	HB3	H	0.62	0.02	2
233	19	PHE	HD1	H	6.12	0.02	1
234	19	PHE	HD2	H	6.12	0.02	1
235	19	PHE	HE1	H	6.30	0.02	1
236	19	PHE	HE2	H	6.30	0.02	1
237	19	PHE	HZ	H	6.37	0.02	1
238	19	PHE	C	C	172.3	0.6	1
239	19	PHE	CA	C	57.0	0.6	1
240	19	PHE	CB	C	41.7	0.6	1
247	19	PHE	N	N	132.0	0.6	1
248	20	ARG	H	H	7.76	0.02	1
249	20	ARG	HA	H	4.83	0.02	1
250	20	ARG	HB2	H	1.26	0.02	2
251	20	ARG	HB3	H	0.99	0.02	2
252	20	ARG	HG2	H	1.59	0.02	2
253	20	ARG	HG3	H	1.42	0.02	2
254	20	ARG	HD2	H	3.35	0.02	2
255	20	ARG	HD3	H	3.10	0.02	2
256	20	ARG	HE	H	7.18	0.02	1
257	20	ARG	HH11	H	6.23	0.02	5
258	20	ARG	HH12	H	6.23	0.02	5
259	20	ARG	HH21	H	6.23	0.02	5
260	20	ARG	HH22	H	6.23	0.02	5
261	20	ARG	C	C	174.7	0.6	1
262	20	ARG	CA	C	54.3	0.6	1
263	20	ARG	CB	C	32.3	0.6	1
264	20	ARG	CG	C	28.0	0.6	1
265	20	ARG	CD	C	44.1	0.6	1
267	20	ARG	N	N	129.4	0.3	1
268	20	ARG	NE	N	85.0	0.3	1
269	20	ARG	NH1	N	70.1	0.3	5
270	20	ARG	NH2	N	70.1	0.3	5
271	21	HIS	H	H	9.30	0.02	1
272	21	HIS	HA	H	4.50	0.02	1
273	21	HIS	HB2	H	3.52	0.02	2
274	21	HIS	HB3	H	3.44	0.02	2

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276	21	HIS	HD2	H	7.02	0.02	1
277	21	HIS	HE1	H	8.44	0.02	1
279	21	HIS	C	C	177.6	0.6	1
280	21	HIS	CA	C	56.4	0.6	1
281	21	HIS	CB	C	32.2	0.6	1
285	21	HIS	N	N	125.9	0.3	1
288	22	LEU	H	H	9.30	0.02	1
289	22	LEU	HA	H	4.11	0.02	1
290	22	LEU	HB2	H	1.87	0.02	1
291	22	LEU	HB3	H	1.65	0.02	1
292	22	LEU	HG	H	1.87	0.02	1
293	22	LEU	HD1	H	0.77	0.02	2
294	22	LEU	HD2	H	0.98	0.02	2
295	22	LEU	C	C	177.7	0.6	1
296	22	LEU	CA	C	57.7	0.6	1
297	22	LEU	CB	C	40.8	0.6	1
298	22	LEU	CG	C	27.6	0.6	1
299	22	LEU	CD1	C	22.6	0.6	2
300	22	LEU	CD2	C	25.3	0.6	2
301	22	LEU	N	N	122.0	0.3	1
302	23	ASP	H	H	7.86	0.02	1
303	23	ASP	HA	H	4.52	0.02	1
304	23	ASP	HB2	H	3.10	0.02	1
305	23	ASP	HB3	H	2.53	0.02	1
306	23	ASP	C	C	176.8	0.6	1
307	23	ASP	CA	C	53.6	0.6	1
308	23	ASP	CB	C	39.7	0.6	1
310	23	ASP	N	N	116.9	0.3	1
311	24	GLU	H	H	8.01	0.02	1
312	24	GLU	HA	H	3.63	0.02	1
313	24	GLU	HB2	H	2.57	0.02	2
314	24	GLU	HB3	H	2.17	0.02	2
315	24	GLU	HG2	H	2.14	0.02	1
316	24	GLU	HG3	H	2.14	0.02	1
317	24	GLU	C	C	176.1	0.6	1
318	24	GLU	CA	C	59.4	0.6	1
319	24	GLU	CB	C	27.5	0.6	1
320	24	GLU	CG	C	37.0	0.6	1
322	24	GLU	N	N	110.3	0.3	1
323	25	ARG	H	H	8.04	0.02	1
324	25	ARG	HA	H	4.24	0.02	1
325	25	ARG	HB2	H	1.84	0.02	2
326	25	ARG	HB3	H	1.75	0.02	2
327	25	ARG	HG2	H	1.56	0.02	1
328	25	ARG	HG3	H	1.56	0.02	1
329	25	ARG	HD2	H	3.16	0.02	1
330	25	ARG	HD3	H	3.16	0.02	1
331	25	ARG	HE	H	8.01	0.02	1
332	25	ARG	HH11	H	6.71	0.02	5
333	25	ARG	HH12	H	6.71	0.02	5
334	25	ARG	HH21	H	6.71	0.02	5
335	25	ARG	HH22	H	6.71	0.02	5
336	25	ARG	C	C	175.6	0.6	1
337	25	ARG	CA	C	57.8	0.6	1
338	25	ARG	CB	C	31.0	0.6	1
339	25	ARG	CG	C	28.3	0.6	1
340	25	ARG	CD	C	43.5	0.6	1
342	25	ARG	N	N	121.9	0.3	1

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343	25	ARG	NE	N	85.8	0.3	1
344	25	ARG	NH1	N	70.3	0.3	5
345	25	ARG	NH2	N	70.3	0.3	5
346	26	GLU	H	H	8.69	0.02	1
347	26	GLU	HA	H	5.40	0.02	1
348	26	GLU	HB2	H	1.90	0.02	1
349	26	GLU	HB3	H	1.90	0.02	1
350	26	GLU	HG2	H	2.63	0.02	2
351	26	GLU	HG3	H	2.09	0.02	2
352	26	GLU	C	C	173.6	0.6	1
353	26	GLU	CA	C	55.6	0.6	1
354	26	GLU	CB	C	37.9	0.6	1
355	26	GLU	CG	C	31.5	0.6	1
357	26	GLU	N	N	124.3	0.3	1
358	27	GLU	H	H	9.07	0.02	1
359	27	GLU	HA	H	4.84	0.02	1
360	27	GLU	HB2	H	2.15	0.02	2
361	27	GLU	HB3	H	2.27	0.02	2
362	27	GLU	HG2	H	2.48	0.02	2
363	27	GLU	HG3	H	2.32	0.02	2
364	27	GLU	C	C	174.5	0.6	1
365	27	GLU	CA	C	54.6	0.6	1
366	27	GLU	CB	C	34.8	0.6	1
367	27	GLU	CG	C	36.5	0.6	1
369	27	GLU	N	N	123.9	0.3	1
370	28	CYS	H	H	8.88	0.02	1
371	28	CYS	HA	H	5.65	0.02	1
372	28	CYS	HB2	H	3.00	0.02	2
373	28	CYS	HB3	H	2.81	0.02	2
375	28	CYS	C	C	175.5	0.6	1
376	28	CYS	CA	C	53.0	0.6	1
377	28	CYS	CB	C	40.9	0.6	1
378	28	CYS	N	N	122.0	0.3	1
379	29	LYS	H	H	8.76	0.02	1
380	29	LYS	HA	H	4.56	0.02	1
381	29	LYS	HB2	H	1.40	0.02	1
382	29	LYS	HB3	H	1.40	0.02	1
383	29	LYS	HG2	H	1.38	0.02	1
384	29	LYS	HG3	H	1.38	0.02	1
385	29	LYS	HD2	H	1.13	0.02	2
386	29	LYS	HD3	H	0.92	0.02	2
387	29	LYS	HE2	H	2.86	0.02	1
388	29	LYS	HE3	H	2.86	0.02	1
389	29	LYS	HZ	H	7.23	0.02	5
390	29	LYS	C	C	174.6	0.6	1
391	29	LYS	CA	C	56.4	0.6	1
392	29	LYS	CB	C	38.4	0.6	1
393	29	LYS	CG	C	25.8	0.6	1
394	29	LYS	CD	C	29.6	0.6	1
395	29	LYS	CE	C	42.4	0.6	1
396	29	LYS	N	N	124.0	0.3	1
397	29	LYS	NZ	N	33.0	0.3	5
398	30	CYS	H	H	8.78	0.02	1
399	30	CYS	HA	H	4.65	0.02	1
400	30	CYS	HB2	H	2.49	0.02	1
401	30	CYS	HB3	H	3.01	0.02	1
403	30	CYS	C	C	173.3	0.6	1
404	30	CYS	CA	C	54.6	0.6	1

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405	30	CYS	CB	C	35.9	0.6	1
406	30	CYS	N	N	121.7	0.3	1
407	31	LEU	H	H	7.81	0.02	1
408	31	LEU	HA	H	4.23	0.02	1
409	31	LEU	HB2	H	1.39	0.02	1
410	31	LEU	HB3	H	1.73	0.02	1
411	31	LEU	HG	H	0.94	0.02	1
412	31	LEU	HD1	H	0.68	0.02	1
413	31	LEU	HD2	H	0.77	0.02	1
414	31	LEU	C	C	176.2	0.6	1
415	31	LEU	CA	C	54.8	0.6	1
416	31	LEU	CB	C	42.5	0.6	1
417	31	LEU	CG	C	27.1	0.6	1
418	31	LEU	CD1	C	26.0	0.6	1
419	31	LEU	CD2	C	21.9	0.6	1
420	31	LEU	N	N	119.2	0.3	1
421	32	LEU	H	H	8.91	0.02	1
422	32	LEU	HA	H	4.26	0.02	1
423	32	LEU	HB2	H	1.75	0.02	1
424	32	LEU	HB3	H	1.32	0.02	1
425	32	LEU	HG	H	1.77	0.02	1
426	32	LEU	HD1	H	0.96	0.02	1
427	32	LEU	HD2	H	0.77	0.02	1
428	32	LEU	C	C	179.0	0.6	1
429	32	LEU	CA	C	56.4	0.6	1
430	32	LEU	CB	C	42.1	0.6	1
431	32	LEU	CG	C	26.9	0.6	1
432	32	LEU	CD1	C	26.0	0.6	1
433	32	LEU	CD2	C	23.4	0.6	1
434	32	LEU	N	N	118.4	0.3	1
435	33	ASN	H	H	8.84	0.02	1
436	33	ASN	HA	H	3.90	0.02	1
437	33	ASN	HB2	H	3.26	0.02	1
438	33	ASN	HB3	H	2.88	0.02	1
439	33	ASN	HD21	H	6.86	0.02	1
440	33	ASN	HD22	H	7.25	0.02	1
441	33	ASN	C	C	173.8	0.6	1
442	33	ASN	CA	C	56.9	0.6	1
443	33	ASN	CB	C	37.3	0.6	1
445	33	ASN	N	N	110.8	0.3	1
446	33	ASN	ND2	N	112.0	0.3	1
447	34	TYR	H	H	8.61	0.02	1
448	34	TYR	HA	H	5.02	0.02	1
449	34	TYR	HB2	H	3.35	0.02	1
450	34	TYR	HB3	H	2.52	0.02	1
451	34	TYR	HD1	H	6.74	0.02	1
452	34	TYR	HD2	H	6.74	0.02	1
453	34	TYR	HE1	H	6.69	0.02	1
454	34	TYR	HE2	H	6.69	0.02	1
456	34	TYR	C	C	174.7	0.6	1
457	34	TYR	CA	C	57.6	0.6	1
458	34	TYR	CB	C	41.0	0.6	1
465	34	TYR	N	N	118.6	0.3	1
466	35	LYS	H	H	9.99	0.02	1
467	35	LYS	HA	H	4.84	0.02	1
468	35	LYS	HB2	H	1.82	0.02	1
469	35	LYS	HB3	H	1.59	0.02	1
470	35	LYS	HG2	H	1.18	0.02	1

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471	35	LYS	HG3	H	1.18	0.02	1
472	35	LYS	HD2	H	1.48	0.02	1
473	35	LYS	HD3	H	1.48	0.02	1
474	35	LYS	HE2	H	2.89	0.02	1
475	35	LYS	HE3	H	2.89	0.02	1
477	35	LYS	C	C	174.1	0.6	1
478	35	LYS	CA	C	54.2	0.6	1
479	35	LYS	CB	C	36.3	0.6	1
480	35	LYS	CG	C	24.1	0.6	1
481	35	LYS	CD	C	29.6	0.6	1
482	35	LYS	CE	C	41.6	0.6	1
483	35	LYS	N	N	119.7	0.3	1
485	36	GLN	H	H	8.77	0.02	1
486	36	GLN	HA	H	4.67	0.02	1
487	36	GLN	HB2	H	2.06	0.02	1
488	36	GLN	HB3	H	2.06	0.02	1
489	36	GLN	HG2	H	2.35	0.02	1
490	36	GLN	HG3	H	2.35	0.02	1
491	36	GLN	HE21	H	7.50	0.02	2
492	36	GLN	HE22	H	6.55	0.02	2
493	36	GLN	C	C	176.3	0.6	1
494	36	GLN	CA	C	56.7	0.6	1
495	36	GLN	CB	C	28.6	0.6	1
496	36	GLN	CG	C	33.7	0.6	1
498	36	GLN	N	N	124.9	0.3	1
499	36	GLN	NE2	N	110.9	0.3	1
500	37	GLU	H	H	8.96	0.02	1
501	37	GLU	HA	H	4.51	0.02	1
502	37	GLU	HB2	H	1.96	0.02	2
503	37	GLU	HB3	H	1.80	0.02	2
504	37	GLU	HG2	H	2.11	0.02	1
505	37	GLU	HG3	H	2.11	0.02	1
506	37	GLU	C	C	176.2	0.6	1
507	37	GLU	CA	C	56.0	0.6	1
508	37	GLU	CB	C	31.9	0.6	1
509	37	GLU	CG	C	36.6	0.6	1
511	37	GLU	N	N	130.2	0.3	1
512	38	GLY	H	H	9.20	0.02	1
513	38	GLY	HA2	H	3.69	0.02	1
514	38	GLY	HA3	H	4.05	0.02	1
515	38	GLY	C	C	174.7	0.6	1
516	38	GLY	CA	C	47.4	0.6	1
517	38	GLY	N	N	118.4	0.3	1
518	39	ASP	H	H	8.81	0.02	1
519	39	ASP	HA	H	4.69	0.02	1
520	39	ASP	HB2	H	2.78	0.02	1
521	39	ASP	HB3	H	2.78	0.02	1
522	39	ASP	C	C	175.2	0.6	1
523	39	ASP	CA	C	54.2	0.6	1
524	39	ASP	CB	C	41.0	0.6	1
526	39	ASP	N	N	127.1	0.3	1
527	40	LYS	H	H	7.86	0.02	1
528	40	LYS	HA	H	4.71	0.02	1
529	40	LYS	HB2	H	1.86	0.02	1
530	40	LYS	HB3	H	1.86	0.02	1
531	40	LYS	HG2	H	1.52	0.02	1
532	40	LYS	HG3	H	1.52	0.02	1
533	40	LYS	HD2	H	1.69	0.02	1

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534	40	LYS	HD3	H	1.69	0.02	1
535	40	LYS	HE2	H	3.02	0.02	1
536	40	LYS	HE3	H	3.02	0.02	1
538	40	LYS	C	C	175.4	0.6	1
539	40	LYS	CA	C	54.8	0.6	1
540	40	LYS	CB	C	36.3	0.6	1
541	40	LYS	CG	C	24.9	0.6	1
542	40	LYS	CD	C	29.0	0.6	1
543	40	LYS	CE	C	42.0	0.6	1
544	40	LYS	N	N	118.0	0.3	1
546	41	CYS	H	H	8.95	0.02	1
547	41	CYS	HA	H	5.29	0.02	1
548	41	CYS	HB2	H	3.00	0.02	1
549	41	CYS	HB3	H	2.58	0.02	1
551	41	CYS	C	C	174.4	0.6	1
552	41	CYS	CA	C	55.0	0.6	1
553	41	CYS	CB	C	41.9	0.6	1
554	41	CYS	N	N	119.8	0.3	1
555	42	VAL	H	H	9.35	0.02	1
556	42	VAL	HA	H	4.88	0.02	1
557	42	VAL	HB	H	2.32	0.02	1
558	42	VAL	HG1	H	1.00	0.02	1
559	42	VAL	HG2	H	0.88	0.02	1
560	42	VAL	C	C	175.6	0.6	1
561	42	VAL	CA	C	59.2	0.6	1
562	42	VAL	CB	C	35.2	0.6	1
563	42	VAL	CG1	C	21.6	0.6	1
564	42	VAL	CG2	C	19.3	0.6	1
565	42	VAL	N	N	118.7	0.3	1
566	43	GLU	H	H	9.18	0.02	1
567	43	GLU	HA	H	3.34	0.02	1
568	43	GLU	HB2	H	1.84	0.02	2
569	43	GLU	HB3	H	1.75	0.02	2
570	43	GLU	HG2	H	2.15	0.02	2
571	43	GLU	HG3	H	2.07	0.02	2
572	43	GLU	C	C	175.4	0.6	1
573	43	GLU	CA	C	59.0	0.6	1
574	43	GLU	CB	C	29.8	0.6	1
575	43	GLU	CG	C	36.8	0.6	1
577	43	GLU	N	N	123.4	0.3	1
578	44	ASN	H	H	8.25	0.02	1
579	44	ASN	HA	H	4.95	0.02	1
580	44	ASN	HB2	H	2.72	0.02	2
581	44	ASN	HB3	H	1.97	0.02	2
582	44	ASN	HD21	H	8.07	0.02	2
583	44	ASN	HD22	H	7.24	0.02	2
584	44	ASN	C	C	?	?	?
585	44	ASN	CA	C	48.5	0.6	1
586	44	ASN	CB	C	39.2	0.6	1
588	44	ASN	N	N	120.2	0.3	1
589	44	ASN	ND2	N	112.1	0.3	1
590	45	PRO	HA	H	4.40	0.02	1
591	45	PRO	HB2	H	2.31	0.02	1
592	45	PRO	HB3	H	1.95	0.02	1
593	45	PRO	HG2	H	1.97	0.02	1
594	45	PRO	HG3	H	1.97	0.02	1
595	45	PRO	HD2	H	3.86	0.02	2
596	45	PRO	HD3	H	3.81	0.02	2

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597	45	PRO	C	C	176.2	0.6	1
598	45	PRO	CA	C	63.7	0.6	1
599	45	PRO	CB	C	32.6	0.6	1
600	45	PRO	CG	C	26.8	0.6	1
601	45	PRO	CD	C	50.9	0.6	1
603	46	ASN	H	H	7.47	0.02	1
604	46	ASN	HA	H	5.09	0.02	1
605	46	ASN	HB2	H	2.72	0.02	2
606	46	ASN	HB3	H	2.43	0.02	2
607	46	ASN	HD21	H	7.57	0.02	1
608	46	ASN	HD22	H	6.91	0.02	1
609	46	ASN	C	C	?	?	?
610	46	ASN	CA	C	51.6	0.6	1
611	46	ASN	CB	C	40.1	0.6	1
613	46	ASN	N	N	114.0	0.3	1
614	46	ASN	ND2	N	112.6	0.3	1
615	47	PRO	HA	H	4.34	0.02	1
616	47	PRO	HB2	H	1.97	0.02	1
617	47	PRO	HB3	H	1.77	0.02	1
618	47	PRO	HG2	H	1.96	0.02	1
619	47	PRO	HG3	H	1.96	0.02	1
620	47	PRO	HD2	H	3.52	0.02	2
621	47	PRO	HD3	H	3.39	0.02	2
622	47	PRO	C	C	175.5	0.6	1
623	47	PRO	CA	C	63.7	0.6	1
624	47	PRO	CB	C	32.4	0.6	1
625	47	PRO	CG	C	27.5	0.6	1
626	47	PRO	CD	C	49.8	0.6	1
628	48	THR	H	H	8.28	0.02	1
629	48	THR	HA	H	4.68	0.02	1
630	48	THR	HB	H	4.32	0.02	1
632	48	THR	HG2	H	1.13	0.02	1
633	48	THR	C	C	174.5	0.6	1
634	48	THR	CA	C	59.1	0.6	1
635	48	THR	CB	C	69.9	0.6	1
636	48	THR	CG2	C	19.3	0.6	1
637	48	THR	N	N	112.4	0.3	1
638	49	CYS	H	H	9.35	0.02	1
639	49	CYS	HA	H	4.44	0.02	1
640	49	CYS	HB2	H	2.64	0.02	1
641	49	CYS	HB3	H	3.14	0.02	1
643	49	CYS	C	C	175.8	0.6	1
644	49	CYS	CA	C	55.5	0.6	1
645	49	CYS	CB	C	37.7	0.6	1
646	49	CYS	N	N	125.3	0.3	1
647	50	ASN	H	H	8.36	0.02	1
648	50	ASN	HA	H	4.60	0.02	1
649	50	ASN	HB2	H	2.88	0.02	1
650	50	ASN	HB3	H	2.70	0.02	1
651	50	ASN	HD21	H	7.60	0.02	2
652	50	ASN	HD22	H	6.97	0.02	2
653	50	ASN	C	C	174.4	0.6	1
654	50	ASN	CA	C	54.8	0.6	1
655	50	ASN	CB	C	38.9	0.6	1
657	50	ASN	N	N	116.4	0.3	1
658	50	ASN	ND2	N	113.6	0.3	1
659	51	GLU	H	H	7.43	0.02	1
660	51	GLU	HA	H	4.64	0.02	1

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661	51	GLU	HB2	H	1.96	0.02	2
662	51	GLU	HB3	H	1.81	0.02	2
663	51	GLU	HG2	H	2.12	0.02	1
664	51	GLU	HG3	H	2.12	0.02	1
665	51	GLU	C	C	176.1	0.6	1
666	51	GLU	CA	C	55.3	0.6	1
667	51	GLU	CB	C	31.5	0.6	1
668	51	GLU	CG	C	36.1	0.6	1
670	51	GLU	N	N	119.2	0.3	1
671	52	ASN	H	H	9.57	0.02	1
672	52	ASN	HA	H	4.45	0.02	1
673	52	ASN	HB2	H	3.14	0.02	1
674	52	ASN	HB3	H	2.64	0.02	1
675	52	ASN	HD21	H	6.99	0.02	1
676	52	ASN	HD22	H	7.71	0.02	1
677	52	ASN	C	C	176.4	0.6	1
678	52	ASN	CA	C	54.2	0.6	1
679	52	ASN	CB	C	37.8	0.6	1
681	52	ASN	N	N	125.4	0.3	1
682	52	ASN	ND2	N	112.8	0.3	1
683	53	ASN	H	H	9.31	0.02	1
684	53	ASN	HA	H	4.64	0.02	1
685	53	ASN	HB2	H	3.25	0.02	1
686	53	ASN	HB3	H	2.31	0.02	1
687	53	ASN	HD21	H	6.95	0.02	1
688	53	ASN	HD22	H	6.26	0.02	1
689	53	ASN	C	C	176.2	0.6	1
690	53	ASN	CA	C	54.1	0.6	1
691	53	ASN	CB	C	39.2	0.6	1
693	53	ASN	N	N	119.3	0.3	1
694	53	ASN	ND2	N	111.6	0.3	1
695	54	GLY	H	H	7.92	0.02	1
696	54	GLY	HA2	H	4.28	0.02	1
697	54	GLY	HA3	H	3.67	0.02	1
698	54	GLY	C	C	173.0	0.6	1
699	54	GLY	CA	C	46.3	0.6	1
700	54	GLY	N	N	106.3	0.3	1
701	55	GLY	H	H	8.06	0.02	1
702	55	GLY	HA2	H	4.39	0.02	1
703	55	GLY	HA3	H	3.47	0.02	1
704	55	GLY	C	C	175.1	0.6	1
705	55	GLY	CA	C	44.4	0.6	1
706	55	GLY	N	N	105.3	0.3	1
707	56	CYS	H	H	7.77	0.02	1
708	56	CYS	HA	H	4.39	0.02	1
709	56	CYS	HB2	H	3.12	0.02	1
710	56	CYS	HB3	H	2.81	0.02	1
712	56	CYS	C	C	175.2	0.6	1
713	56	CYS	CA	C	53.1	0.6	1
714	56	CYS	CB	C	36.6	0.6	1
715	56	CYS	N	N	117.6	0.3	1
716	57	ASP	H	H	8.37	0.02	1
717	57	ASP	HA	H	3.98	0.02	1
718	57	ASP	HB2	H	2.24	0.02	2
719	57	ASP	HB3	H	2.03	0.02	2
720	57	ASP	C	C	176.0	0.6	1
721	57	ASP	CA	C	56.0	0.6	1
722	57	ASP	CB	C	45.0	0.6	1



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724	57	ASP	N	N	122.3	0.3	1
725	58	ALA	H	H	8.48	0.02	1
726	58	ALA	HA	H	3.98	0.02	1
727	58	ALA	HB	H	1.41	0.02	1
728	58	ALA	C	C	178.9	0.6	1
729	58	ALA	CA	C	55.6	0.6	1
730	58	ALA	CB	C	19.1	0.6	1
731	58	ALA	N	N	127.2	0.3	1
732	59	ASP	H	H	9.11	0.02	1
733	59	ASP	HA	H	4.90	0.02	1
734	59	ASP	HB2	H	2.44	0.02	1
735	59	ASP	HB3	H	2.82	0.02	1
736	59	ASP	C	C	174.6	0.6	1
737	59	ASP	CA	C	53.9	0.6	1
738	59	ASP	CB	C	40.7	0.6	1
740	59	ASP	N	N	116.7	0.3	1
741	60	ALA	H	H	7.94	0.02	1
742	60	ALA	HA	H	5.13	0.02	1
743	60	ALA	HB	H	1.27	0.02	1
744	60	ALA	C	C	176.3	0.6	1
745	60	ALA	CA	C	50.5	0.6	1
746	60	ALA	CB	C	21.3	0.6	1
747	60	ALA	N	N	121.9	0.3	1
748	61	LYS	H	H	9.06	0.02	1
749	61	LYS	HA	H	4.54	0.02	1
750	61	LYS	HB2	H	1.77	0.02	1
751	61	LYS	HB3	H	1.77	0.02	1
752	61	LYS	HG2	H	1.31	0.02	2
753	61	LYS	HG3	H	1.39	0.02	2
754	61	LYS	HD2	H	1.72	0.02	2
755	61	LYS	HD3	H	1.63	0.02	2
756	61	LYS	HE2	H	2.92	0.02	2
757	61	LYS	HE3	H	2.92	0.02	2
759	61	LYS	C	C	175.7	0.6	1
760	61	LYS	CA	C	55.1	0.6	1
761	61	LYS	CB	C	34.1	0.6	1
762	61	LYS	CG	C	24.6	0.6	1
763	61	LYS	CD	C	29.1	0.6	1
764	61	LYS	CE	C	42.1	0.6	1
765	61	LYS	N	N	122.5	0.3	1
767	62	CYS	H	H	9.19	0.02	1
768	62	CYS	HA	H	5.32	0.02	1
769	62	CYS	HB2	H	2.44	0.02	1
770	62	CYS	HB3	H	2.80	0.02	1
772	62	CYS	C	C	174.5	0.6	1
773	62	CYS	CA	C	56.1	0.6	1
774	62	CYS	CB	C	37.6	0.6	1
775	62	CYS	N	N	131.5	0.3	1
776	63	THR	H	H	9.23	0.02	1
777	63	THR	HA	H	4.51	0.02	1
778	63	THR	HB	H	4.01	0.02	1
780	63	THR	HG2	H	1.16	0.02	1
781	63	THR	C	C	172.2	0.6	1
782	63	THR	CA	C	62.8	0.6	1
783	63	THR	CB	C	71.5	0.6	1
784	63	THR	CG2	C	22.0	0.6	1
785	63	THR	N	N	125.9	0.3	1
786	64	GLU	H	H	8.61	0.02	1

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787	64	GLU	HA	H	5.12	0.02	1
788	64	GLU	HB2	H	1.90	0.02	1
789	64	GLU	HB3	H	1.90	0.02	1
790	64	GLU	HG2	H	2.30	0.02	1
791	64	GLU	HG3	H	2.30	0.02	1
792	64	GLU	C	C	?	?	?
793	64	GLU	CA	C	54.4	0.6	1
794	64	GLU	CB	C	32.0	0.6	1
795	64	GLU	CG	C	36.8	0.6	1
797	64	GLU	N	N	123.0	0.3	1
798	65	GLU	H	H	8.76	0.02	1
799	65	GLU	HA	H	4.60	0.02	1
800	65	GLU	HB2	H	2.01	0.02	2
801	65	GLU	HB3	H	1.86	0.02	2
802	65	GLU	HG2	H	2.15	0.02	1
803	65	GLU	HG3	H	2.15	0.02	1
804	65	GLU	C	C	?	?	?
805	65	GLU	CA	C	55.1	0.6	1
806	65	GLU	CB	C	32.9	0.6	1
807	65	GLU	CG	C	36.1	0.6	1
809	65	GLU	N	N	123.0	0.3	1
810	66	ASP	H	H	8.79	0.02	1
811	66	ASP	HA	H	4.80	0.02	1
812	66	ASP	HB2	H	2.80	0.02	2
813	66	ASP	HB3	H	2.58	0.02	2
814	66	ASP	C	C	176.4	0.6	1
815	66	ASP	CA	C	54.8	0.6	1
816	66	ASP	CB	C	41.2	0.6	1
818	66	ASP	N	N	123.9	0.3	1
819	67	SER	H	H	8.38	0.02	1
820	67	SER	HA	H	4.55	0.02	1
821	67	SER	HB2	H	3.83	0.02	2
822	67	SER	HB3	H	3.70	0.02	2
824	67	SER	C	C	175.4	0.6	1
825	67	SER	CA	C	58.0	0.6	1
826	67	SER	CB	C	64.6	0.6	1
827	67	SER	N	N	119.1	0.3	1
828	68	GLY	H	H	8.65	0.02	1
829	68	GLY	HA2	H	4.13	0.02	2
830	68	GLY	HA3	H	3.85	0.02	2
831	68	GLY	C	C	175.0	0.6	1
832	68	GLY	CA	C	46.0	0.6	1
833	68	GLY	N	N	112.2	0.3	1
834	69	SER	H	H	8.58	0.02	1
835	69	SER	HA	H	4.42	0.02	1
836	69	SER	HB2	H	3.87	0.02	1
837	69	SER	HB3	H	3.87	0.02	1
839	69	SER	C	C	174.8	0.6	1
840	69	SER	CA	C	59.1	0.6	1
841	69	SER	CB	C	63.4	0.6	1
842	69	SER	N	N	117.7	0.3	1
843	70	ASN	H	H	8.39	0.02	1
844	70	ASN	HA	H	4.74	0.02	1
845	70	ASN	HB2	H	2.94	0.02	2
846	70	ASN	HB3	H	2.77	0.02	2
847	70	ASN	HD21	H	?	?	?
848	70	ASN	HD22	H	?	?	?
849	70	ASN	C	C	?	?	?

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850	70	ASN	CA	C	53.8	0.6	1
851	70	ASN	CB	C	38.9	0.6	1
853	70	ASN	N	N	118.4	0.3	1
854	70	ASN	ND2	N	?	?	?
855	71	GLY	H	H	7.88	0.02	1
856	71	GLY	HA2	H	4.07	0.02	1
857	71	GLY	HA3	H	4.07	0.02	1
858	71	GLY	C	C	173.7	0.6	1
859	71	GLY	CA	C	45.0	0.6	1
860	71	GLY	N	N	108.1	0.3	1
861	72	LYS	H	H	8.39	0.02	1
862	72	LYS	HA	H	4.84	0.02	1
863	72	LYS	HB2	H	1.75	0.02	2
864	72	LYS	HB3	H	1.59	0.02	2
865	72	LYS	HG2	H	1.46	0.02	2
866	72	LYS	HG3	H	1.38	0.02	2
867	72	LYS	HD2	H	1.66	0.02	1
868	72	LYS	HD3	H	1.66	0.02	1
869	72	LYS	HE2	H	2.93	0.02	1
870	72	LYS	HE3	H	2.93	0.02	1
872	72	LYS	C	C	173.7	0.6	1
873	72	LYS	CA	C	55.4	0.6	1
874	72	LYS	CB	C	35.0	0.6	1
875	72	LYS	CG	C	24.9	0.6	1
876	72	LYS	CD	C	28.9	0.6	1
877	72	LYS	CE	C	42.2	0.6	1
878	72	LYS	N	N	120.8	0.3	1
880	73	LYS	H	H	8.81	0.02	1
881	73	LYS	HA	H	4.64	0.02	1
882	73	LYS	HB2	H	1.71	0.02	1
883	73	LYS	HB3	H	1.71	0.02	1
884	73	LYS	HG2	H	1.36	0.02	2
885	73	LYS	HG3	H	1.25	0.02	2
886	73	LYS	HD2	H	1.63	0.02	1
887	73	LYS	HD3	H	1.63	0.02	1
888	73	LYS	HE2	H	2.89	0.02	1
889	73	LYS	HE3	H	2.89	0.02	1
891	73	LYS	C	C	175.3	0.6	1
892	73	LYS	CA	C	55.0	0.6	1
893	73	LYS	CB	C	35.4	0.6	1
894	73	LYS	CG	C	24.6	0.6	1
895	73	LYS	CD	C	?	?	?
896	73	LYS	CE	C	?	?	?
897	73	LYS	N	N	122.6	0.3	1
899	74	ILE	H	H	8.54	0.02	1
900	74	ILE	HA	H	4.83	0.02	1
901	74	ILE	HB	H	1.91	0.02	1
902	74	ILE	HG12	H	1.50	0.02	2
903	74	ILE	HG13	H	1.50	0.02	2
904	74	ILE	HG2	H	1.00	0.02	1
905	74	ILE	HD1	H	0.57	0.02	1
906	74	ILE	C	C	176.2	0.6	1
907	74	ILE	CA	C	58.4	0.6	1
908	74	ILE	CB	C	38.4	0.6	1
909	74	ILE	CG1	C	27.1	0.6	1
910	74	ILE	CG2	C	19.0	0.6	1
911	74	ILE	CD1	C	10.3	0.6	1
912	74	ILE	N	N	126.8	0.3	1

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913	75	THR	H	H	8.88	0.02	1
914	75	THR	HA	H	4.51	0.02	1
915	75	THR	HB	H	4.01	0.02	1
917	75	THR	HG2	H	1.15	0.02	1
918	75	THR	C	C	172.4	0.6	1
919	75	THR	CA	C	61.2	0.6	1
920	75	THR	CB	C	71.5	0.6	1
921	75	THR	CG2	C	22.0	0.6	1
922	75	THR	N	N	120.3	0.3	1
923	76	CYS	H	H	8.82	0.02	1
924	76	CYS	HA	H	5.53	0.02	1
925	76	CYS	HB2	H	3.28	0.02	1
926	76	CYS	HB3	H	2.69	0.02	1
928	76	CYS	C	C	174.7	0.6	1
929	76	CYS	CA	C	52.2	0.6	1
930	76	CYS	CB	C	40.6	0.6	1
931	76	CYS	N	N	121.5	0.3	1
932	77	GLU	H	H	8.64	0.02	1
933	77	GLU	HA	H	4.74	0.02	1
934	77	GLU	HB2	H	1.94	0.02	1
935	77	GLU	HB3	H	1.94	0.02	1
936	77	GLU	HG2	H	1.83	0.02	1
937	77	GLU	HG3	H	1.83	0.02	1
938	77	GLU	C	C	175.7	0.6	1
939	77	GLU	CA	C	54.7	0.6	1
940	77	GLU	CB	C	33.1	0.6	1
941	77	GLU	CG	C	36.1	0.6	1
943	77	GLU	N	N	123.0	0.3	1
944	78	CYS	H	H	9.71	0.02	1
945	78	CYS	HA	H	4.48	0.02	1
946	78	CYS	HB2	H	2.63	0.02	1
947	78	CYS	HB3	H	3.30	0.02	1
949	78	CYS	C	C	175.8	0.6	1
950	78	CYS	CA	C	57.4	0.6	1
951	78	CYS	CB	C	39.2	0.6	1
952	78	CYS	N	N	129.1	0.3	1
953	79	THR	H	H	8.20	0.02	1
954	79	THR	HA	H	4.25	0.02	1
955	79	THR	HB	H	4.25	0.02	1
957	79	THR	HG2	H	1.25	0.02	1
958	79	THR	C	C	176.2	0.6	1
959	79	THR	CA	C	63.4	0.6	1
960	79	THR	CB	C	70.1	0.6	1
961	79	THR	CG2	C	22.2	0.6	1
962	79	THR	N	N	114.7	0.3	1
963	80	LYS	H	H	8.56	0.02	1
964	80	LYS	HA	H	4.50	0.02	1
965	80	LYS	HB2	H	1.79	0.02	1
966	80	LYS	HB3	H	1.79	0.02	1
967	80	LYS	HG2	H	1.47	0.02	2
968	80	LYS	HG3	H	1.64	0.02	2
969	80	LYS	HD2	H	1.70	0.02	1
970	80	LYS	HD3	H	1.70	0.02	1
971	80	LYS	HE2	H	2.94	0.02	1
972	80	LYS	HE3	H	2.94	0.02	1
974	80	LYS	C	C	?	?	?
975	80	LYS	CA	C	55.4	0.6	1
976	80	LYS	CB	C	30.9	0.6	1

977	80	LYS	CG	C	25.6	0.6	1
978	80	LYS	CD	C	29.1	0.6	1
979	80	LYS	CE	C	42.1	0.6	1
980	80	LYS	N	N	124.8	0.3	1
982	81	PRO	HA	H	4.26	0.02	1
983	81	PRO	HB2	H	2.27	0.02	1
984	81	PRO	HB3	H	1.87	0.02	1
985	81	PRO	HG2	H	2.10	0.02	2
986	81	PRO	HG3	H	2.04	0.02	2
987	81	PRO	HD2	H	3.90	0.02	2
988	81	PRO	HD3	H	3.63	0.02	2
989	81	PRO	C	C	176.9	0.6	1
990	81	PRO	CA	C	64.2	0.6	1
991	81	PRO	CB	C	31.9	0.6	1
992	81	PRO	CG	C	27.8	0.6	1
993	81	PRO	CD	C	50.8	0.6	1
995	82	ASP	H	H	8.78	0.02	1
996	82	ASP	HA	H	4.25	0.02	1
997	82	ASP	HB2	H	2.88	0.02	2
998	82	ASP	HB3	H	2.77	0.02	2
999	82	ASP	C	C	?	?	?
1000	82	ASP	CA	C	55.1	0.6	1
1001	82	ASP	CB	C	40.0	0.6	1
1003	82	ASP	N	N	117.9	0.3	1
1004	83	SER	H	H	7.39	0.02	1
1005	83	SER	HA	H	4.47	0.02	1
1006	83	SER	HB2	H	3.55	0.02	2
1007	83	SER	HB3	H	3.50	0.02	2
1009	83	SER	C	C	175.4	0.6	1
1010	83	SER	CA	C	57.4	0.6	1
1011	83	SER	CB	C	65.9	0.6	1
1012	83	SER	N	N	112.9	0.3	1
1013	84	TYR	H	H	8.72	0.02	1
1014	84	TYR	HA	H	5.01	0.02	1
1015	84	TYR	HB2	H	2.93	0.02	2
1016	84	TYR	HB3	H	2.75	0.02	2
1017	84	TYR	HD1	H	6.92	0.02	1
1018	84	TYR	HD2	H	6.92	0.02	1
1019	84	TYR	HE1	H	6.69	0.02	1
1020	84	TYR	HE2	H	6.69	0.02	1
1022	84	TYR	C	C	?	?	?
1023	84	TYR	CA	C	54.6	0.6	1
1024	84	TYR	CB	C	39.7	0.6	1
1031	84	TYR	N	N	122.2	0.3	1
1032	85	PRO	HA	H	5.09	0.02	1
1033	85	PRO	HB2	H	2.25	0.02	2
1034	85	PRO	HB3	H	1.72	0.02	2
1035	85	PRO	HG2	H	2.22	0.02	1
1036	85	PRO	HG3	H	2.22	0.02	1
1037	85	PRO	HD2	H	3.85	0.02	1
1038	85	PRO	HD3	H	3.85	0.02	1
1039	85	PRO	C	C	176.9	0.6	1
1040	85	PRO	CA	C	62.9	0.6	1
1041	85	PRO	CB	C	32.8	0.6	1
1042	85	PRO	CG	C	27.3	0.6	1
1043	85	PRO	CD	C	50.6	0.6	1
1045	86	LEU	H	H	8.51	0.02	1
1046	86	LEU	HA	H	4.77	0.02	1

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1047	86	LEU	HB2	H	1.76	0.02	1
1048	86	LEU	HB3	H	1.76	0.02	1
1049	86	LEU	HG	H	1.85	0.02	1
1050	86	LEU	HD1	H	1.00	0.02	1
1051	86	LEU	HD2	H	1.00	0.02	1
1052	86	LEU	C	C	177.9	0.6	1
1053	86	LEU	CA	C	54.5	0.6	1
1054	86	LEU	CB	C	44.8	0.6	1
1055	86	LEU	CG	C	29.0	0.6	1
1056	86	LEU	CD1	C	25.7	0.6	1
1057	86	LEU	CD2	C	25.7	0.6	1
1058	86	LEU	N	N	120.2	0.3	1
1059	87	PHE	H	H	9.29	0.02	1
1060	87	PHE	HA	H	4.16	0.02	1
1061	87	PHE	HB2	H	3.26	0.02	2
1062	87	PHE	HB3	H	3.19	0.02	2
1063	87	PHE	HD1	H	7.31	0.02	1
1064	87	PHE	HD2	H	7.31	0.02	1
1065	87	PHE	HE1	H	7.70	0.02	1
1066	87	PHE	HE2	H	7.70	0.02	1
1067	87	PHE	HZ	H	7.67	0.02	1
1068	87	PHE	C	C	176.4	0.6	1
1069	87	PHE	CA	C	59.3	0.6	1
1070	87	PHE	CB	C	36.9	0.6	1
1077	87	PHE	N	N	122.6	0.3	1
1078	88	ASP	H	H	8.93	0.02	1
1079	88	ASP	HA	H	4.33	0.02	1
1080	88	ASP	HB2	H	3.11	0.02	2
1081	88	ASP	HB3	H	3.02	0.02	2
1082	88	ASP	C	C	175.2	0.6	1
1083	88	ASP	CA	C	56.3	0.6	1
1084	88	ASP	CB	C	39.9	0.6	1
1086	88	ASP	N	N	111.3	0.3	1
1087	89	GLY	H	H	7.87	0.02	1
1088	89	GLY	HA2	H	3.48	0.02	1
1089	89	GLY	HA3	H	4.08	0.02	1
1090	89	GLY	C	C	174.7	0.6	1
1091	89	GLY	CA	C	46.0	0.6	1
1092	89	GLY	N	N	102.0	0.3	1
1093	90	ILE	H	H	7.17	0.02	1
1094	90	ILE	HA	H	4.39	0.02	1
1095	90	ILE	HB	H	1.52	0.02	1
1096	90	ILE	HG12	H	0.65	0.02	2
1097	90	ILE	HG13	H	0.65	0.02	2
1098	90	ILE	HG2	H	1.05	0.02	1
1099	90	ILE	HD1	H	0.51	0.02	1
1100	90	ILE	C	C	175.1	0.6	1
1101	90	ILE	CA	C	64.2	0.6	1
1102	90	ILE	CB	C	35.9	0.6	1
1103	90	ILE	CG1	C	25.5	0.6	1
1104	90	ILE	CG2	C	16.9	0.6	1
1105	90	ILE	CD1	C	14.9	0.6	1
1106	90	ILE	N	N	113.3	0.3	1
1107	91	PHE	H	H	7.43	0.02	1
1108	91	PHE	HA	H	5.15	0.02	1
1109	91	PHE	HB2	H	2.40	0.02	1
1110	91	PHE	HB3	H	2.40	0.02	1
1111	91	PHE	HD1	H	7.00	0.02	1

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1112	91	PHE	HD2	H	7.00	0.02	1
1113	91	PHE	HE1	H	6.95	0.02	1
1114	91	PHE	HE2	H	6.95	0.02	1
1115	91	PHE	HZ	H	7.06	0.02	1
1116	91	PHE	C	C	175.1	0.6	1
1117	91	PHE	CA	C	56.5	0.6	1
1118	91	PHE	CB	C	44.3	0.6	1
1125	91	PHE	N	N	114.0	0.3	1
1126	92	CYS	H	H	7.86	0.02	1
1127	92	CYS	HA	H	5.18	0.02	1
1128	92	CYS	HB2	H	2.57	0.02	1
1129	92	CYS	HB3	H	3.08	0.02	1
1131	92	CYS	C	C	174.1	0.6	1
1132	92	CYS	CA	C	54.1	0.6	1
1133	92	CYS	CB	C	44.7	0.6	1
1134	92	CYS	N	N	119.0	0.3	1
1135	93	SER	H	H	9.26	0.02	1
1136	93	SER	HA	H	4.23	0.02	1
1137	93	SER	HB2	H	3.90	0.02	1
1138	93	SER	HB3	H	3.90	0.02	1
1140	93	SER	C	C	?	?	?
1141	93	SER	CA	C	60.4	0.6	1
1142	93	SER	CB	C	63.7	0.6	1
1143	93	SER	N	N	118.3	0.3	1
1144	94	SER	H	H	8.04	0.02	1
1145	94	SER	HA	H	4.59	0.02	1
1146	94	SER	HB2	H	3.91	0.02	1
1147	94	SER	HB3	H	3.91	0.02	1
1149	94	SER	C	C	?	?	?
1150	94	SER	CA	C	58.7	0.6	1
1151	94	SER	CB	C	64.0	0.6	1
1152	94	SER	N	N	114.2	0.3	1
1153	95	SER	H	H	?	?	?
1154	95	SER	HA	H	4.71	0.02	1
1155	95	SER	HB2	H	4.02	0.02	2
1156	95	SER	HB3	H	3.94	0.02	2
1158	95	SER	C	C	?	?	?
1159	95	SER	CA	C	58.4	0.6	1
1160	95	SER	CB	C	64.5	0.6	1
1161	95	SER	N	N	?	?	?
1162	96	ASN	H	H	?	?	?
1163	96	ASN	HA	H	4.61	0.02	1
1164	96	ASN	HB2	H	2.75	0.02	2
1165	96	ASN	HB3	H	2.60	0.02	2
1166	96	ASN	HD21	H	?	?	?
1167	96	ASN	HD22	H	?	?	?
1168	96	ASN	C	C	?	?	?
1169	96	ASN	CA	C	54.3	0.6	1
1170	96	ASN	CB	C	41.8	0.6	1
1172	96	ASN	N	N	?	?	?
1173	96	ASN	ND2	N	?	?	?

stop\_

# The following loop is used to define sets of Atom-shift assignment IDs that represent related ambiguous assignments taken from the above list of assigned chemical shifts. Each element in the set should be separated by a comma, as shown in the example below, and is the assignment ID for a chemical shift

assignment that has been given as ambiguity code of 4 or 5. Each set indicates that the observed chemical shifts are related to the defined atoms, but have not been assigned uniquely to a specific atom in the set.

```
loop_  
  _Atom_shift_assign_ID_ambiguity  
  
#  
#   Sets of Atom-shift Assignment Ambiguities  
#       #  
#   -----  
# Example:  5,4,7  
#  
#           @  
stop_
```



```
#####
##          ----REMARKS----          ##
#####
#   #   #   #   #   #   #   #   #   #
#
# PROTECTED BACKBONE AMIDE GROUPS
# (SLOWLY EXCHANGING IN D2O) FOR RESIDUES:
# GLY 17 , PHE 19 , GLU 27 , LYS 29 , LEU 31 ,
# TYR 34 , LYS 35 , VAL 42 , CYS 56 , ASP 57 ,
# ALA 60 , LYS 61 , THR 63 , THR 75 , GLU 77 ,
# LEU 86 , GLY 89 , ILE 90 , PHE 91
#
# BROAD HN SIGNALS IN [15-N]-HSQC OBSERVED FOR RESIDUES:
# VAL 8 , LYS 9 , LYS 10 , CYS 18 , ARG 20
#
# TWO BACKBONE HN CROSSPEAKS OBSERVED FOR RESIDUES:
# HIS 5 : 7.78,113.8 / 7.74,113.5
# GLN 6 : 7.44,122.6 / 7.40,122.4
#
# TWO AVERAGED NH*/HH* SIGNALS OBSERVED FOR RESIDUES:
# ARG 20 , ARG 25 : NOT SPECIFICALLY ASSIGNED
# TO INDIVIDUAL ARGININES
#
# LYS 29 NZ/HZ* SIGNAL: TENTATIVELY ASSIGNED TO
# LYS 29 (BURIED LYSINE SIDE CHAIN)
# BASED ON GREATER PROTECTION FROM H2O EXCHANGE
# THAN OTHER LYSINE NZ/HZ* SIGNALS
#
# ASPARAGINE SIDE CHAIN AMIDE SIGNALS:
# PROBABLE OVERLAPPING CROSSPEAKS ~112 PPM [15N]
# FOR ASN 1 , ASN 70 , ASN 96
#
#
#####
#                                     #
#####
```

Table A2. Supplementary: NMR experimental details

Experiment	Dimension	Nucleus	Complex Points [after LP] (points)	Spectral width (Hz)	Acquisition Time (ms)	Carrier Frequency (ppm)	Instrument <sup>1</sup> H- frequency (MHz)	Solvent	Temp- erature (°C)	Final data size (points)	Digital Resolution (Hz/ point)	Mixing time (ms)	Total time (hr)
2D NOESY	t1	<sup>1</sup> H	400	8000	50	4.74	600	D <sub>2</sub> O	25	1024	7.8	75-150	22
	t2	<sup>1</sup> H	2048	8000	256	4.74				2048	3.9		
2D NOESY	t1	<sup>1</sup> H	360	8000	45	4.74	600	H <sub>2</sub> O	25	1024	7.8	75	23
	t2	<sup>1</sup> H	2048	8000	256	4.74				2048	3.9		
2D ROESY	t1	<sup>1</sup> H	260	6000	43	4.74	500	D <sub>2</sub> O	25	1024	5.9	60	16
	t2	<sup>1</sup> H	2048	6000	341	4.74				2048	2.9		
2D ROESY	t1	<sup>1</sup> H	360	7000	54	4.74	500	H <sub>2</sub> O	25	1024	6.8	60	62
	t2	<sup>1</sup> H	2048	7000	293	4.74				2048	3.4		
3D [ <sup>15</sup> N]-NOESY-HSQ	t1	<sup>15</sup> N	36 [64]	2500	14.4	121.5	500	H <sub>2</sub> O	25	128	19.5	125	64
	t2	<sup>1</sup> H	180	7000	26	4.74				512	13.7		
	t3	<sup>1</sup> H	512	7000	73	4.74				512	13.7		
3D [ <sup>15</sup> N]-ROESY-HSQC	t1	<sup>15</sup> N	32	2500	12.8	121.5	500	H <sub>2</sub> O	25	128	19.5	60	87
	t2	<sup>1</sup> H	180	7000	26	4.74				512	13.7		
	t3	<sup>1</sup> H	512	7000	73	4.74				512	13.7		
3D [ <sup>13</sup> C]-HMQC-NOESY	t1	<sup>1</sup> H	160	7200	22	4.74	600	D <sub>2</sub> O	25	512	14.1	125	89
	t2	<sup>13</sup> C	96	10000	9.6	41.0				256	39.1		
	t3	<sup>1</sup> H	384	7200	53	4.74				1024	7.0		
4D [ <sup>13</sup> C]-HMQC-NOESY- <sup>13</sup> C]-HSQC	t1	<sup>13</sup> C	18 [24]	3360	5.4	40.2	600	D <sub>2</sub> O	25	64	52.5	125	105
	t2	<sup>1</sup> H	74	3600	21	3.00				256	14.1		
	t3	<sup>13</sup> C	18 [24]	3360	5.4	40.2				64	52.5		
	t4	<sup>1</sup> H	256	4500	57	3.00				256	17.6		

Experiment (Reference)	Dimension	Nucleus	Complex Points [after LP] (points)	Spectral width (Hz)	Acquisition Time (ms)	Carrier Frequency (ppm)	Instrument <sup>1</sup> H-frequency (MHz)	Solvent	Temper- ature (°C)	Final data size (points)	Digital Res. (Hz/ point)(ms)	Mixing time (hr)	Total time (hr)
4D [ <sup>13</sup> C]-HMQC-NOESY-[( <sup>15</sup> N)-HSQC													
	t1	<sup>13</sup> C	14 [24]	3360	4.2	40.2	600	H <sub>2</sub> O	25	64	52.5	125	96
	t2	<sup>1</sup> H	56	3600	15.6	3.00				128	28.1		
	t3	<sup>15</sup> N	14 [24]	1800	7.8	118.9				64	28.1		
	t4	<sup>1</sup> H	256	7400	34.6	4.74				256	29.0		
2D DQF-COSY													
	t1	<sup>1</sup> H	1000	6000	167	4.74	500	D <sub>2</sub> O	25	4096	1.5		31
	t2	<sup>1</sup> H	2048	6000	341	4.74				8192	0.7		
3D HNHA													
	t1	<sup>15</sup> N	35	2500	14	121.5	500	H <sub>2</sub> O	25	128	19.5		41
	t2	<sup>1</sup> H	80	3500	23	4.74				256	13.7		
	t3	<sup>1</sup> H	512	7000	73	4.74				512	13.7		
3D HNHB													
	t1	<sup>15</sup> N	24 [48]	2500	9.6	119.1	600	H <sub>2</sub> O	25	128	19.5		60
	t2	<sup>1</sup> H	90	8000	11.3	4.74				256	31.3		
	t3	<sup>1</sup> H	512	8000	64	4.74				1024	7.8		
3D HN(CO)HB													
	t1	<sup>15</sup> N	28 [48]	1800	15.6	118.9	600	H <sub>2</sub> O	25	128	14.1		108
	t2	<sup>1</sup> H	128	8000	16	4.74				512	15.6		
	t3	<sup>1</sup> H	512	8000	64	4.74				512	15.6		
2D [ <sup>15</sup> N]-[ <sup>13</sup> C] Spin-echo HSQC													
	t1	<sup>15</sup> N	80	1800	44.4	118.9	600	H <sub>2</sub> O	25	256	7.0		13
	t2	<sup>1</sup> H	1312	8000	149	4.74				2048	3.9		
2D [ <sup>13</sup> C]-[ <sup>13</sup> C] Spin-echo HSQC													
	t1	<sup>15</sup> N	78	1800	43.3	118.9	600	H <sub>2</sub> O	25	256	7.0		26
	t2	<sup>1</sup> H	1216	8000	152	4.74				2048	3.9		
3D LRCH													
	t1	<sup>13</sup> C	34	3017	11.3	17.9	600	D <sub>2</sub> O	25	256	11.8		84
	t2	<sup>1</sup> H	57	4800	11.9	2.25				256	18.8		
	t3	<sup>1</sup> H	384	4000	96	2.25				1024	3.9		

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Experiment {Reference}	Dimension	Nucleus	Complex Points	Spectral width [after LP] (Hz)	Acquisition Time (ms)	Carrier Frequency (ppm)	Instrument <sup>1</sup> H-frequency (MHz)	Solvent	Temper- ature (°C)	Final data size (points)	Digital Resolution (Hz/ point)	Mixing time (ms)	Total time (hr)
2D TOCSY	t1	<sup>1</sup> H	360	8000	45	4.74	600	H <sub>2</sub> O	25	1024	7.8	66	18
	t2	<sup>1</sup> H	2048	8000	256	4.74				2048	3.9		
2D [ <sup>15</sup> N]-HSQC	t1	<sup>15</sup> N	360	4400	82	119.6	600	H <sub>2</sub> O	25	2048	2.1		14
	t2	<sup>1</sup> H	1216	8000	152	4.74				4096	2.0		
2D [ <sup>13</sup> C]-HSQC	t1	<sup>13</sup> C	400	12000	33.3	41.3	600	H <sub>2</sub> O	25	1024	11.7		2.7
	t2	<sup>1</sup> H	1216	8000	152	4.74				4096	2.0		
3D [ <sup>15</sup> N]-TOCSY-HSQ	t1	<sup>15</sup> N	38 [64]	2500	15.2	119.0	600	H <sub>2</sub> O	25	128	19.5	56	43
	t2	<sup>1</sup> H	180	8000	22.5	4.74				512	15.6		
	t3	<sup>1</sup> H	512	8000	64	4.74				512	15.6		
3D HCCH-TOCSY	t1	<sup>1</sup> H	134	5500	24.4	4.74	500	D <sub>2</sub> O	25	512	10.7	17	65
	t2	<sup>13</sup> C	128	8049	15.9	41.9				512	15.7		
	t3	<sup>1</sup> H	416	5500	76	4.74				512	10.7		
3D CBCA(CO)NH	t1	<sup>13</sup> C	38 [64]	10000	3.8	41.3	600	H <sub>2</sub> O	25	128	78.1		21
	t2	<sup>15</sup> N	26 [36]	1800	14.4	118.9				128	14.1		
	t3	<sup>1</sup> H	512	8000	64	4.74				512	15.6		
3D CBCANH	t1	<sup>13</sup> C	63 [128]	10000	6.3	41.3	600	H <sub>2</sub> O	25	512	19.5		5
	t2	<sup>15</sup> N	26 [52]	1800	14.4	118.9				128	14.1		
	t3	<sup>1</sup> H	512	8000	64	4.74				256	31.3		
3D HNCO	t1	<sup>15</sup> N	32 [48]	1800	17.8	118.9	600	H <sub>2</sub> O	25	128	14.1		43
	t2	<sup>13</sup> C	64	1811	35.3	176.0				256	7.1		
	t3	<sup>1</sup> H	512	8000	64	4.74				512	15.6		

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TABLE B

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! merozoite surface protein-1 (MSP-1) P. falciparum C-terminal fragment
! X-PLOR format
! 09-11-98
! --- noes + roes --approximate-- 570 total
!     long-range      185
!     medium-range    90
!     sequential      222
!     intraresidue     73
!     hydrogen_bonds  10 (20 restraints)
!
! pseudoatom corrections not used -- for R-6 averaging/summation
! AVERAGING:
! class rt6s SUM
! class nsam SUM
! class sing
! class hbnd
! types:
!     arom_          aromatic pair
!     meth_          methyl
!     dgnm_          degenerate methylene
!     nsam_          non-stereospecifically-assigned methylene
!     sing_          single proton
!     _l             long-range          (j-i > 4)
!     _m             medium-range        (j-i =2-4)
!     _s             sequential          (j-i = 1)
!     _i             intraresidue
!     hbnd_          hydrogen_bonds
!
!     <residue-atom 1>          <residue-atom 2>          <dist-minus-plus>
! <type>
!
class rt6s

assign (resid 5 and name hb#) (resid 19 and name hd#) 3.6 3.6 0.0 !arom_l
assign (resid 5 and name hb#) (resid 19 and name he#) 3.6 3.6 0.0 !arom_l
assign (resid 15 and name ha) (resid 34 and name hd#) 3.6 3.6 0.0 !arom_l
assign (resid 15 and name ha) (resid 34 and name he#) 3.6 3.6 0.0 !arom_l
assign (resid 17 and name ha#) (resid 87 and name hd#) 5.5 5.5 0.0 !arom_l
assign (resid 17 and name ha#) (resid 87 and name he#) 5.5 5.5 0.0 !arom_l
assign (resid 19 and name hd#) (resid 20 and name hn) 5.5 5.5 0.0 !arom_s
assign (resid 19 and name hd#) (resid 21 and name hn) 5.5 5.5 0.0 !arom_s
assign (resid 19 and name hd#) (resid 21 and name hd2) 3.6 3.6 0.0 !arom_m
assign (resid 19 and name hd#) (resid 22 and name hd#) 3.6 3.6 0.0 !arom_m
assign (resid 19 and name hd#) (resid 86 and name hg) 5.5 5.5 0.0 !arom_l
assign (resid 19 and name hd#) (resid 91 and name hb#) 3.6 3.6 0.0 !arom_l
assign (resid 19 and name hd#) (resid 91 and name hd#) 3.6 3.6 0.0 !arom_l
assign (resid 19 and name hd#) (resid 91 and name he#) 5.5 5.5 0.0 !arom_l
assign (resid 19 and name he#) (resid 21 and name hd2) 5.5 5.5 0.0 !arom_m
assign (resid 19 and name he#) (resid 22 and name hd#) 3.6 3.6 0.0 !arom_m
assign (resid 19 and name he#) (resid 86 and name hg) 5.5 5.5 0.0 !arom_l
assign (resid 19 and name he#) (resid 91 and name hb#) 3.6 3.6 0.0 !arom_l
assign (resid 19 and name he#) (resid 91 and name hd#) 5.5 5.5 0.0 !arom_l
assign (resid 31 and name hn) (resid 34 and name hd#) 3.6 3.6 0.0 !arom_m
assign (resid 31 and name hg) (resid 34 and name hd#) 5.5 5.5 0.0 !arom_m
assign (resid 31 and name hd#) (resid 34 and name hd#) 5.5 5.5 0.0 !arom_m
assign (resid 31 and name hd1#) (resid 34 and name he#) 5.5 5.5 0.0 !arom_m
assign (resid 31 and name hd2#) (resid 87 and name hd#) 2.8 2.8 0.0 !arom_l
assign (resid 32 and name hn) (resid 34 and name hd#) 5.5 5.5 0.0 !arom_m
assign (resid 34 and name hn) (resid 34 and name hd#) 3.6 3.6 0.0 !arom_i
assign (resid 34 and name ha) (resid 34 and name hd#) 3.6 3.6 0.0 !arom_i
assign (resid 34 and name hd#) (resid 35 and name hn) 3.6 3.6 0.0 !arom_s

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assign (resid 34 and name hd#)	(resid 42 and name hn)	5.5	5.5	0.0	!arom_l
assign (resid 34 and name hd#)	(resid 42 and name ha)	3.6	3.6	0.0	!arom_l
assign (resid 34 and name he#)	(resid 43 and name hn)	5.5	5.5	0.0	!arom_l
assign (resid 34 and name hd#)	(resid 43 and name hb#)	5.5	5.5	0.0	!arom_l
assign (resid 34 and name he#)	(resid 42 and name hn)	5.5	5.5	0.0	!arom_l
assign (resid 34 and name he#)	(resid 42 and name ha)	5.5	5.5	0.0	!arom_l
assign (resid 34 and name he#)	(resid 43 and name hb#)	5.5	5.5	0.0	!arom_l
assign (resid 34 and name he#)	(resid 43 and name hg#)	3.6	3.6	0.0	!arom_l
assign (resid 83 and name hn)	(resid 84 and name hd#)	3.6	3.6	0.0	!arom_s
assign (resid 86 and name hn)	(resid 91 and name hd#)	5.5	5.5	0.0	!arom_l
assign (resid 86 and name hb#)	(resid 87 and name hd#)	2.8	2.8	0.0	!arom_s
assign (resid 86 and name hb#)	(resid 87 and name he#)	3.6	3.6	0.0	!arom_s
assign (resid 86 and name hd#)	(resid 87 and name hd#)	3.6	3.6	0.0	!arom_s
assign (resid 86 and name hd#)	(resid 87 and name he#)	3.6	3.6	0.0	!arom_s
assign (resid 87 and name ha)	(resid 87 and name hd#)	2.8	2.8	0.0	!arom_i
assign (resid 87 and name hd#)	(resid 90 and name hb)	5.5	5.5	0.0	!arom_m
assign (resid 87 and name hd#)	(resid 90 and name hg2#)	5.5	5.5	0.0	!arom_m
assign (resid 87 and name hd#)	(resid 90 and name hd#)	3.6	3.6	0.0	!arom_m
assign (resid 87 and name hd#)	(resid 91 and name hb#)	3.6	3.6	0.0	!arom_m
assign (resid 87 and name hd#)	(resid 91 and name hd#)	2.8	2.8	0.0	!arom_m
assign (resid 87 and name hd#)	(resid 91 and name he#)	3.6	3.6	0.0	!arom_m
assign (resid 87 and name he#)	(resid 91 and name hd#)	5.5	5.5	0.0	!arom_m
assign (resid 87 and name he#)	(resid 91 and name he#)	5.5	5.5	0.0	!arom_m
assign (resid 91 and name hn)	(resid 91 and name hd#)	3.6	3.6	0.0	!arom_i
assign (resid 91 and name hd#)	(resid 92 and name hn)	5.5	5.5	0.0	!arom_s
assign (resid 2 and name hd#)	(resid 86 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 5 and name hb#)	(resid 86 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 17 and name hn)	(resid 90 and name hd#)	4.1	4.1	0.0	!meth_l
assign (resid 17 and name ha#)	(resid 90 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 29 and name hb#)	(resid 90 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 29 and name hg#)	(resid 90 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 29 and name hd#)	(resid 90 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 29 and name he#)	(resid 90 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 31 and name hn)	(resid 90 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 31 and name ha)	(resid 90 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 31 and name ha)	(resid 90 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 32 and name ha)	(resid 90 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 32 and name hb#)	(resid 90 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 32 and name hg)	(resid 90 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 40 and name hn)	(resid 40 and name he#)	5.5	5.5	0.0	!meth_i
assign (resid 47 and name ha)	(resid 48 and name hg2#)	5.5	5.5	0.0	!meth_s
assign (resid 47 and name hb1)	(resid 74 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 47 and name hg#)	(resid 74 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 48 and name hn)	(resid 48 and name hg2#)	3.6	3.6	0.0	!meth_i
assign (resid 48 and name hn)	(resid 74 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 48 and name ha)	(resid 74 and name hd#)	3.6	3.6	0.0	!meth_l
assign (resid 48 and name hg2#)	(resid 49 and name hn)	5.5	5.5	0.0	!meth_s
assign (resid 48 and name hg2#)	(resid 50 and name hn)	3.6	3.6	0.0	!meth_m
assign (resid 48 and name hg2#)	(resid 50 and name hb1)	5.5	5.5	0.0	!meth_m
assign (resid 48 and name hg2#)	(resid 51 and name hn)	3.1	3.1	0.0	!meth_m
assign (resid 48 and name hg2#)	(resid 51 and name ha)	3.6	3.6	0.0	!meth_m
assign (resid 48 and name hg2#)	(resid 51 and name hb#)	3.6	3.6	0.0	!meth_m
assign (resid 48 and name hg2#)	(resid 51 and name hg#)	5.5	5.5	0.0	!meth_m
assign (resid 48 and name hg2#)	(resid 74 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 49 and name hn)	(resid 74 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 49 and name ha)	(resid 74 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 54 and name hn)	(resid 74 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 54 and name ha#)	(resid 74 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 54 and name ha#)	(resid 74 and name hd#)	5.5	5.5	0.0	!meth_l
assign (resid 56 and name hn)	(resid 60 and name hb#)	5.5	5.5	0.0	!meth_m
assign (resid 56 and name hn)	(resid 74 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 56 and name ha)	(resid 60 and name hb#)	5.5	5.5	0.0	!meth_m
assign (resid 56 and name ha)	(resid 90 and name hg2#)	5.5	5.5	0.0	!meth_l
assign (resid 56 and name hb1)	(resid 60 and name hb#)	3.6	3.6	0.0	!meth_m
assign (resid 57 and name hn)	(resid 60 and name hb#)	3.6	3.6	0.0	!meth_m

assign (resid 57 and name ha)	(resid 60 and name hb#)	5.5 5.5 0.0	!meth_m
assign (resid 57 and name hb#)	(resid 60 and name hb#)	5.5 5.5 0.0	!meth_m
assign (resid 58 and name hb#)	(resid 59 and name hn)	3.6 3.6 0.0	!meth_s
assign (resid 58 and name hb#)	(resid 60 and name hn)	5.5 5.5 0.0	!meth_m
assign (resid 59 and name hn)	(resid 60 and name hb#)	5.5 5.5 0.0	!meth_s
assign (resid 60 and name hb#)	(resid 61 and name hn)	3.6 3.6 0.0	!meth_s
assign (resid 60 and name hb#)	(resid 76 and name ha)	5.5 5.5 0.0	!meth_l
assign (resid 60 and name hb#)	(resid 76 and name hb1)	5.5 5.5 0.0	!meth_l
assign (resid 60 and name hb#)	(resid 77 and name hn)	3.6 3.6 0.0	!meth_l
assign (resid 60 and name hb#)	(resid 77 and name ha)	5.5 5.5 0.0	!meth_l
assign (resid 60 and name hb#)	(resid 78 and name hn)	5.5 5.5 0.0	!meth_l
assign (resid 60 and name hb#)	(resid 78 and name hb1)	5.5 5.5 0.0	!meth_l
assign (resid 62 and name ha)	(resid 74 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 63 and name hn)	(resid 74 and name hg2#)	4.1 4.1 0.0	!meth_l
assign (resid 63 and name hn)	(resid 74 and name hd#)	3.6 3.6 0.0	!meth_l
assign (resid 63 and name hg2#)	(resid 64 and name hn)	3.6 3.6 0.0	!meth_s
assign (resid 64 and name ha)	(resid 74 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 64 and name ha)	(resid 74 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 64 and name hb#)	(resid 74 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 64 and name hg#)	(resid 74 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 72 and name hn)	(resid 72 and name he#)	3.6 3.6 0.0	!meth_i
assign (resid 72 and name hb#)	(resid 74 and name hd#)	5.5 5.5 0.0	!meth_m
assign (resid 73 and name hn)	(resid 74 and name hd#)	5.5 5.5 0.0	!meth_s
assign (resid 74 and name hn)	(resid 74 and name hg2#)	3.6 3.6 0.0	!meth_i
assign (resid 74 and name ha)	(resid 74 and name hg2#)	3.6 3.6 0.0	!meth_i
assign (resid 74 and name ha)	(resid 74 and name hd#)	3.6 3.6 0.0	!meth_i
assign (resid 74 and name hg2#)	(resid 75 and name ha)	5.5 5.5 0.0	!meth_s
assign (resid 75 and name hg2#)	(resid 76 and name hn)	3.6 3.6 0.0	!meth_s
assign (resid 79 and name hn)	(resid 79 and name hg2#)	3.6 3.6 0.0	!meth_i
assign (resid 79 and name hg2#)	(resid 80 and name hn)	3.6 3.6 0.0	!meth_s
assign (resid 85 and name ha)	(resid 86 and name hd#)	5.5 5.5 0.0	!meth_s
assign (resid 86 and name hd#)	(resid 87 and name hn)	5.5 5.5 0.0	!meth_s
assign (resid 86 and name hd#)	(resid 91 and name hb#)	5.5 5.5 0.0	!meth_l
assign (resid 86 and name hd#)	(resid 92 and name ha)	5.5 5.5 0.0	!meth_l
assign (resid 87 and name ha)	(resid 90 and name hd#)	5.5 5.5 0.0	!meth_m
assign (resid 87 and name hb#)	(resid 90 and name hg2#)	5.5 5.5 0.0	!meth_m
assign (resid 87 and name hb#)	(resid 90 and name hd#)	5.5 5.5 0.0	!meth_m
assign (resid 89 and name hn)	(resid 90 and name hg2#)	5.5 5.5 0.0	!meth_s
assign (resid 90 and name hn)	(resid 90 and name hg2#)	3.6 3.6 0.0	!meth_i
assign (resid 90 and name ha)	(resid 90 and name hg2#)	3.6 3.6 0.0	!meth_i
assign (resid 90 and name hg2#)	(resid 91 and name hn)	5.5 5.5 0.0	!meth_s
assign (resid 90 and name hd#)	(resid 91 and name hn)	5.5 5.5 0.0	!meth_s
assign (resid 6 and name ha)	(resid 6 and name hg#)	2.8 2.8 0.0	!dgnm_i
assign (resid 10 and name he#)	(resid 19 and name hn)	3.6 3.6 0.0	!dgnm_l
assign (resid 10 and name he#)	(resid 28 and name hn)	3.6 3.6 0.0	!dgnm_l
assign (resid 14 and name hn)	(resid 14 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 14 and name hb#)	(resid 15 and name hn)	4.1 4.1 0.0	!dgnm_s
assign (resid 24 and name hn)	(resid 24 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 24 and name ha)	(resid 24 and name hg#)	2.8 2.8 0.0	!dgnm_i
assign (resid 24 and name hg#)	(resid 25 and name hn)	5.5 5.5 0.0	!dgnm_s
assign (resid 25 and name hn)	(resid 25 and name hd#)	5.5 5.5 0.0	!dgnm_i
assign (resid 26 and name hn)	(resid 26 and name hb#)	2.8 2.8 0.0	!dgnm_i
assign (resid 29 and name hb#)	(resid 29 and name hd#)	3.6 3.6 0.0	!dgnm_i
assign (resid 29 and name hb#)	(resid 30 and name hn)	3.6 3.6 0.0	!dgnm_s
assign (resid 33 and name hn)	(resid 47 and name hg#)	5.5 5.5 0.0	!dgnm_l
assign (resid 35 and name hn)	(resid 35 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 35 and name ha)	(resid 35 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 35 and name hg#)	(resid 36 and name hn)	3.6 3.6 0.0	!dgnm_s
assign (resid 35 and name hg#)	(resid 42 and name hn)	5.5 5.5 0.0	!dgnm_l
assign (resid 35 and name hg#)	(resid 44 and name ha)	3.6 3.6 0.0	!dgnm_l
assign (resid 35 and name hg#)	(resid 44 and name hb#)	3.6 3.6 0.0	!dgnm_l
assign (resid 36 and name hn)	(resid 36 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 36 and name ha)	(resid 36 and name hg#)	2.8 2.8 0.0	!dgnm_i
assign (resid 36 and name hb#)	(resid 37 and name hn)	5.5 5.5 0.0	!dgnm_s
assign (resid 36 and name hg#)	(resid 37 and name hn)	3.6 3.6 0.0	!dgnm_s

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assign (resid 36 and name hg#)	(resid 40 and name hn)	5.5 5.5 0.0	!dgnm_m
assign (resid 37 and name ha)	(resid 37 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 37 and name hg#)	(resid 38 and name hn)	4.1 4.1 0.0	!dgnm_s
assign (resid 40 and name hn)	(resid 40 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 40 and name hn)	(resid 40 and name hd#)	5.5 5.5 0.0	!dgnm_i
assign (resid 44 and name ha)	(resid 45 and name hg#)	3.6 3.6 0.0	!dgnm_s
assign (resid 45 and name hg#)	(resid 46 and name hn)	3.6 3.6 0.0	!dgnm_s
assign (resid 47 and name hg#)	(resid 48 and name hn)	5.5 5.5 0.0	!dgnm_s
assign (resid 49 and name hn)	(resid 51 and name hg#)	3.6 3.6 0.0	!dgnm_m
assign (resid 49 and name hn)	(resid 64 and name hb#)	5.5 5.5 0.0	!dgnm_l
assign (resid 49 and name hn)	(resid 64 and name hg#)	5.5 5.5 0.0	!dgnm_l
assign (resid 51 and name hn)	(resid 51 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 51 and name hg#)	(resid 52 and name hd2#)	5.5 5.5 0.0	!dgnm_s
assign (resid 61 and name hn)	(resid 61 and name hb#)	2.8 2.8 0.0	!dgnm_i
assign (resid 61 and name hb#)	(resid 62 and name hn)	3.6 3.6 0.0	!dgnm_s
assign (resid 64 and name hn)	(resid 64 and name hb#)	2.8 2.8 0.0	!dgnm_i
assign (resid 64 and name hn)	(resid 64 and name hg#)	2.8 2.8 0.0	!dgnm_i
assign (resid 64 and name hn)	(resid 65 and name hg#)	2.8 2.8 0.0	!dgnm_s
assign (resid 64 and name ha)	(resid 64 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 64 and name hb#)	(resid 74 and name ha)	3.6 3.6 0.0	!dgnm_l
assign (resid 65 and name hn)	(resid 65 and name hg#)	3.6 3.6 0.0	!dgnm_i
assign (resid 69 and name hb#)	(resid 70 and name hn)	2.8 2.8 0.0	!dgnm_s
assign (resid 71 and name ha#)	(resid 72 and name hn)	2.6 2.6 0.0	!dgnm_s
assign (resid 72 and name hn)	(resid 72 and name hd#)	3.6 3.6 0.0	!dgnm_i
assign (resid 73 and name hn)	(resid 73 and name hb#)	2.8 2.8 0.0	!dgnm_i
assign (resid 73 and name hb#)	(resid 74 and name hn)	3.6 3.6 0.0	!dgnm_s
assign (resid 80 and name hn)	(resid 80 and name hb#)	2.8 2.8 0.0	!dgnm_i
assign (resid 80 and name hb#)	(resid 81 and name hd#)	2.8 2.8 0.0	!dgnm_s
assign (resid 80 and name hb#)	(resid 83 and name hn)	3.6 3.6 0.0	!dgnm_m
assign (resid 80 and name hb#)	(resid 83 and name hb#)	2.8 2.8 0.0	!dgnm_m
assign (resid 84 and name ha)	(resid 85 and name hg#)	3.6 3.6 0.0	!dgnm_s
assign (resid 84 and name ha)	(resid 85 and name hd#)	2.8 2.8 0.0	!dgnm_s
assign (resid 85 and name hg#)	(resid 89 and name hn)	5.5 5.5 0.0	!dgnm_m
assign (resid 86 and name hb#)	(resid 87 and name hn)	3.6 3.6 0.0	!dgnm_s
assign (resid 86 and name hb#)	(resid 91 and name hb#)	3.6 3.6 0.0	!dgnm_l

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assign (resid 8 and name ha)	(resid 8 and name hg#)	3.6 3.6 0.0	!meth_i
assign (resid 8 and name hg#)	(resid 20 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 8 and name hg#)	(resid 20 and name he)	5.5 5.5 0.0	!meth_l
assign (resid 15 and name ha)	(resid 31 and name hd1#)	3.6 3.6 0.0	!meth_l
assign (resid 15 and name hb#)	(resid 31 and name hd1#)	5.5 5.5 0.0	!meth_l
assign (resid 16 and name hn)	(resid 31 and name hd1#)	5.5 5.5 0.0	!meth_l
assign (resid 16 and name ha)	(resid 31 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 17 and name hn)	(resid 31 and name hd2#)	4.1 4.1 0.0	!meth_l
assign (resid 17 and name ha#)	(resid 31 and name hd2#)	5.5 5.5 0.0	!meth_l
assign (resid 19 and name hz)	(resid 22 and name hd#)	3.6 3.6 0.0	!meth_m
assign (resid 22 and name hd#)	(resid 23 and name hn)	5.5 5.5 0.0	!meth_s
assign (resid 30 and name ha)	(resid 31 and name hd#)	5.5 5.5 0.0	!meth_s
assign (resid 31 and name ha)	(resid 31 and name hd2#)	3.6 3.6 0.0	!meth_i
assign (resid 31 and name hd2#)	(resid 87 and name hb#)	5.5 5.5 0.0	!meth_l
assign (resid 31 and name hd2#)	(resid 87 and name hz)	3.6 3.6 0.0	!meth_l
assign (resid 31 and name hd2#)	(resid 90 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 31 and name hd2#)	(resid 90 and name hd#)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name ha)	(resid 32 and name hd2#)	3.6 3.6 0.0	!meth_i
assign (resid 32 and name hd2#)	(resid 33 and name hn)	3.6 3.6 0.0	!meth_s
assign (resid 32 and name hd2#)	(resid 55 and name ha#)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name hd2#)	(resid 56 and name hn)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name hd2#)	(resid 56 and name ha)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name hd2#)	(resid 74 and name hb)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name hd2#)	(resid 74 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name hd2#)	(resid 90 and name ha)	5.5 5.5 0.0	!meth_l
assign (resid 32 and name hd2#)	(resid 90 and name hg2#)	3.6 3.6 0.0	!meth_l
assign (resid 32 and name hd1#)	(resid 88 and name hb#)	5.5 5.5 0.0	!meth_l



assign (resid 35 and name hn)	(resid 42 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 35 and name hb2)	(resid 42 and name hgl#)	5.5 5.5 0.0	!meth_l
assign (resid 35 and name hg#)	(resid 42 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 37 and name hn)	(resid 42 and name hgl#)	5.5 5.5 0.0	!meth_l
assign (resid 37 and name hn)	(resid 42 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 37 and name ha)	(resid 42 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 37 and name hb#)	(resid 42 and name hgl#)	5.5 5.5 0.0	!meth_l
assign (resid 37 and name hb#)	(resid 42 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 37 and name hg#)	(resid 42 and name hgl#)	5.5 5.5 0.0	!meth_l
assign (resid 37 and name hg#)	(resid 42 and name hg2#)	5.5 5.5 0.0	!meth_l
assign (resid 40 and name hn)	(resid 42 and name hgl#)	5.5 5.5 0.0	!meth_m
assign (resid 40 and name hg#)	(resid 42 and name hgl#)	5.5 5.5 0.0	!meth_m
assign (resid 41 and name ha)	(resid 42 and name hgl#)	5.5 5.5 0.0	!meth_s
assign (resid 41 and name ha)	(resid 42 and name hg2#)	5.5 5.5 0.0	!meth_s
assign (resid 41 and name hb#)	(resid 42 and name hg#)	5.5 5.5 0.0	!meth_s
assign (resid 42 and name hgl#)	(resid 43 and name hn)	3.6 3.6 0.0	!meth_s
assign (resid 42 and name hg2#)	(resid 43 and name hn)	3.6 3.6 0.0	!meth_s
assign (resid 5 and name ha)	(resid 20 and name hb#)	3.6 3.6 0.0	!nsam_l
assign (resid 7 and name ha)	(resid 20 and name hb#)	3.6 3.6 0.0	!nsam_l
assign (resid 7 and name ha)	(resid 20 and name hd#)	3.6 3.6 0.0	!nsam_l
assign (resid 10 and name ha)	(resid 10 and name hg#)	3.6 3.6 0.0	!nsam_i
assign (resid 12 and name ha)	(resid 13 and name hd#)	2.8 2.8 0.0	!nsam_s
assign (resid 12 and name hb#)	(resid 13 and name hd#)	3.6 3.6 0.0	!nsam_s
assign (resid 12 and name hb#)	(resid 28 and name hb#)	3.6 3.6 0.0	!nsam_l
assign (resid 13 and name hb2)	(resid 14 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 13 and name hg#)	(resid 16 and name hb#)	3.6 3.6 0.0	!nsam_m
assign (resid 13 and name hd#)	(resid 28 and name hb#)	3.6 3.6 0.0	!nsam_l
assign (resid 15 and name hb2)	(resid 41 and name hb2)	3.6 3.6 0.0	!sing_l
assign (resid 16 and name ha)	(resid 30 and name hb#)	3.6 3.6 0.0	!nsam_l
assign (resid 16 and name hb#)	(resid 30 and name ha)	3.6 3.6 0.0	!nsam_l
assign (resid 16 and name hb#)	(resid 31 and name hn)	3.6 3.6 0.0	!nsam_l
assign (resid 17 and name ha#)	(resid 87 and name hz)	3.6 3.6 0.0	!nsam_l
assign (resid 20 and name ha)	(resid 20 and name hg#)	3.6 3.6 0.0	!nsam_i
assign (resid 20 and name ha)	(resid 26 and name hg#)	3.6 3.6 0.0	!nsam_l
assign (resid 20 and name hb#)	(resid 20 and name hd#)	3.6 3.6 0.0	!nsam_i
assign (resid 20 and name hg#)	(resid 21 and name hn)	3.6 3.6 0.0	!nsam_s
assign (resid 20 and name hg#)	(resid 24 and name ha)	3.6 3.6 0.0	!nsam_m
assign (resid 21 and name hb#)	(resid 22 and name hn)	5.5 5.5 0.0	!nsam_s
assign (resid 21 and name hb#)	(resid 23 and name hn)	5.5 5.5 0.0	!nsam_m
assign (resid 22 and name hb2)	(resid 23 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 24 and name hb#)	(resid 25 and name hn)	5.5 5.5 0.0	!nsam_s
assign (resid 25 and name hn)	(resid 25 and name hg#)	3.6 3.6 0.0	!nsam_i
assign (resid 25 and name hg#)	(resid 26 and name hn)	3.6 3.6 0.0	!nsam_s
assign (resid 26 and name hn)	(resid 26 and name hg#)	3.6 3.6 0.0	!nsam_i
assign (resid 26 and name ha)	(resid 26 and name hg#)	3.6 3.6 0.0	!nsam_i
assign (resid 27 and name ha)	(resid 27 and name hg#)	2.8 2.8 0.0	!nsam_i
assign (resid 27 and name hb#)	(resid 28 and name hn)	2.8 2.8 0.0	!nsam_s
assign (resid 27 and name hg#)	(resid 28 and name hn)	2.8 2.8 0.0	!nsam_s
assign (resid 28 and name hn)	(resid 28 and name hb#)	2.8 2.8 0.0	!nsam_i
assign (resid 29 and name hg#)	(resid 30 and name hn)	2.8 2.8 0.0	!nsam_s
assign (resid 29 and name hd#)	(resid 90 and name hb)	3.6 3.6 0.0	!nsam_l
assign (resid 30 and name hn)	(resid 30 and name hb#)	2.8 2.8 0.0	!nsam_i
assign (resid 30 and name hbl)	(resid 31 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 30 and name hb#)	(resid 34 and name hn)	5.5 5.5 0.0	!nsam_m
assign (resid 30 and name hb2)	(resid 34 and name hb2)	3.6 3.6 0.0	!sing_m
assign (resid 30 and name hb#)	(resid 35 and name ha)	3.6 3.6 0.0	!nsam_l
assign (resid 31 and name hn)	(resid 31 and name hb#)	3.6 3.6 0.0	!nsam_i
assign (resid 31 and name hn)	(resid 34 and name hb2)	3.6 3.6 0.0	!sing_m
assign (resid 32 and name hbl)	(resid 33 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 33 and name ha)	(resid 44 and name hb#)	3.6 3.6 0.0	!nsam_l
assign (resid 33 and name ha)	(resid 47 and name hbl)	3.6 3.6 0.0	!sing_l
assign (resid 33 and name hb2)	(resid 34 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 34 and name ha)	(resid 44 and name hb#)	3.6 3.6 0.0	!nsam_l
assign (resid 34 and name hbl)	(resid 35 and name hn)	4.1 4.1 0.0	!sing_s
assign (resid 35 and name hb#)	(resid 35 and name hd#)	2.8 2.8 0.0	!nsam_i

assign (resid 35 and name hb2)	(resid 36 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 35 and name hb#)	(resid 44 and name hb#)	3.6 3.6 0.0	!nsam_l
assign (resid 35 and name hd#)	(resid 44 and name ha)	3.6 3.6 0.0	!nsam_l
assign (resid 37 and name hn)	(resid 40 and name hb#)	5.5 5.5 0.0	!nsam_m
assign (resid 38 and name ha#)	(resid 39 and name hn)	3.1 3.1 0.0	!nsam_s
assign (resid 38 and name ha#)	(resid 40 and name hn)	5.5 5.5 0.0	!nsam_m
assign (resid 39 and name hb#)	(resid 40 and name hn)	3.6 3.6 0.0	!nsam_s
assign (resid 41 and name hb1)	(resid 42 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 43 and name hn)	(resid 43 and name hb#)	2.8 2.8 0.0	!nsam_i
assign (resid 43 and name ha)	(resid 43 and name hg#)	2.8 2.8 0.0	!nsam_i
assign (resid 43 and name hb#)	(resid 44 and name hn)	3.6 3.6 0.0	!nsam_s
assign (resid 43 and name hg#)	(resid 44 and name hn)	3.6 3.6 0.0	!nsam_s
assign (resid 44 and name hn)	(resid 45 and name hd#)	5.5 5.5 0.0	!nsam_s
assign (resid 44 and name ha)	(resid 45 and name hd#)	2.8 2.8 0.0	!nsam_s
assign (resid 45 and name hb2)	(resid 46 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 46 and name hn)	(resid 46 and name hb#)	3.6 3.6 0.0	!nsam_i
assign (resid 46 and name hn)	(resid 47 and name hd#)	3.6 3.6 0.0	!nsam_s
assign (resid 46 and name ha)	(resid 47 and name hd#)	3.6 3.6 0.0	!nsam_s
assign (resid 47 and name hb2)	(resid 48 and name hn)	4.1 4.1 0.0	!sing_s
assign (resid 47 and name hb2)	(resid 74 and name hgl#)	3.6 3.6 0.0	!nsam_l
assign (resid 47 and name hg#)	(resid 74 and name hgl#)	3.6 3.6 0.0	!nsam_l
assign (resid 48 and name hn)	(resid 54 and name ha#)	4.1 4.1 0.0	!nsam_l
assign (resid 48 and name hn)	(resid 74 and name hgl#)	5.5 5.5 0.0	!nsam_l
assign (resid 49 and name ha)	(resid 54 and name ha#)	3.6 3.6 0.0	!nsam_l
assign (resid 49 and name ha)	(resid 62 and name hb1)	5.5 5.5 0.0	!sing_l
assign (resid 50 and name hb1)	(resid 51 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 53 and name hb#)	(resid 56 and name hn)	5.5 5.5 0.0	!nsam_m
assign (resid 53 and name hb1)	(resid 58 and name ha)	3.6 3.6 0.0	!sing_l
assign (resid 53 and name hd22)	(resid 56 and name hn)	5.5 5.5 0.0	!sing_m
assign (resid 53 and name hd21)	(resid 60 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 54 and name ha#)	(resid 55 and name hn)	3.6 3.6 0.0	!nsam_s
assign (resid 54 and name ha#)	(resid 56 and name hn)	3.6 3.6 0.0	!nsam_m
assign (resid 55 and name ha#)	(resid 56 and name hn)	3.6 3.6 0.0	!nsam_s
assign (resid 56 and name hb1)	(resid 57 and name hn)	4.1 4.1 0.0	!sing_s
assign (resid 56 and name hb2)	(resid 62 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 56 and name hb2)	(resid 62 and name ha)	3.6 3.6 0.0	!sing_l
assign (resid 57 and name hb#)	(resid 58 and name hn)	3.6 3.6 0.0	!nsam_s
assign (resid 57 and name hb#)	(resid 59 and name hn)	4.1 4.1 0.0	!nsam_m
assign (resid 57 and name hb#)	(resid 60 and name hn)	4.1 4.1 0.0	!nsam_m
assign (resid 57 and name hb#)	(resid 91 and name ha)	2.8 2.8 0.0	!nsam_l
assign (resid 57 and name hb#)	(resid 92 and name hn)	5.5 5.5 0.0	!nsam_l
assign (resid 58 and name hn)	(resid 59 and name hb#)	5.5 5.5 0.0	!nsam_s
assign (resid 59 and name hb#)	(resid 80 and name hg#)	3.6 3.6 0.0	!nsam_l
assign (resid 59 and name hb#)	(resid 60 and name hn)	3.6 3.6 0.0	!nsam_s
assign (resid 60 and name ha)	(resid 78 and name hb#)	3.6 3.6 0.0	!nsam_l
assign (resid 61 and name hn)	(resid 61 and name hg#)	3.6 3.6 0.0	!nsam_i
assign (resid 65 and name hn)	(resid 65 and name hb#)	2.8 2.8 0.0	!nsam_i
assign (resid 66 and name hn)	(resid 66 and name hb#)	2.8 2.8 0.0	!nsam_i
assign (resid 66 and name hb#)	(resid 67 and name hn)	3.6 3.6 0.0	!nsam_s
assign (resid 67 and name hb#)	(resid 73 and name hb#)	3.6 3.6 0.0	!nsam_l
assign (resid 68 and name ha#)	(resid 69 and name hn)	3.6 3.6 0.0	!nsam_s
assign (resid 72 and name hn)	(resid 72 and name hg#)	3.6 3.6 0.0	!nsam_i
assign (resid 74 and name hn)	(resid 74 and name hgl#)	3.6 3.6 0.0	!nsam_i
assign (resid 74 and name ha)	(resid 74 and name hgl#)	3.6 3.6 0.0	!nsam_i
assign (resid 76 and name hn)	(resid 76 and name hb#)	2.8 2.8 0.0	!nsam_i
assign (resid 76 and name hb1)	(resid 77 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 76 and name hb1)	(resid 89 and name ha#)	3.6 3.6 0.0	!nsam_l
assign (resid 78 and name hb1)	(resid 79 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 78 and name hb#)	(resid 92 and name hb#)	3.6 3.6 0.0	!nsam_l
assign (resid 78 and name hb#)	(resid 80 and name hn)	5.5 5.5 0.0	!nsam_m
assign (resid 78 and name hb#)	(resid 83 and name hb#)	3.6 3.6 0.0	!nsam_l
assign (resid 80 and name hn)	(resid 80 and name hg#)	3.6 3.6 0.0	!nsam_i
assign (resid 80 and name hn)	(resid 81 and name hd#)	5.5 5.5 0.0	!nsam_s
assign (resid 80 and name hn)	(resid 83 and name hb#)	3.6 3.6 0.0	!nsam_m
assign (resid 80 and name ha)	(resid 80 and name hg#)	3.6 3.6 0.0	!nsam_i

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assign (resid 80 and name ha)	(resid 81 and name hd#)	2.8 2.8 0.0	!nsam_s
assign (resid 80 and name hg#)	(resid 81 and name hd#)	3.6 3.6 0.0	!nsam_s
assign (resid 81 and name hb2)	(resid 82 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 81 and name hd#)	(resid 82 and name hn)	5.5 5.5 0.0	!nsam_s
assign (resid 82 and name hb#)	(resid 83 and name hn)	3.6 3.6 0.0	!nsam_s
assign (resid 85 and name ha)	(resid 89 and name ha#)	3.6 3.6 0.0	!nsam_m
assign (resid 85 and name hb#)	(resid 88 and name ha)	3.6 3.6 0.0	!nsam_m
assign (resid 85 and name hb#)	(resid 89 and name hn)	3.6 3.6 0.0	!nsam_m
assign (resid 85 and name hb#)	(resid 86 and name hn)	3.6 3.6 0.0	!nsam_s
assign (resid 86 and name hn)	(resid 89 and name ha#)	5.5 5.5 0.0	!nsam_m
assign (resid 87 and name hb#)	(resid 90 and name hn)	3.6 3.6 0.0	!nsam_m
assign (resid 87 and name hb#)	(resid 90 and name hgl#)	5.5 5.5 0.0	!nsam_m
assign (resid 89 and name ha#)	(resid 90 and name hn)	3.6 3.6 0.0	!nsam_s
assign (resid 90 and name hgl#)	(resid 91 and name hn)	4.1 4.1 0.0	!nsam_s

class sing

assign (resid 2 and name hn)	(resid 3 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 4 and name hn)	(resid 5 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 5 and name hn)	(resid 6 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 5 and name ha)	(resid 20 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 6 and name hn)	(resid 7 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 7 and name hn)	(resid 8 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 8 and name hn)	(resid 9 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 9 and name hn)	(resid 10 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 13 and name ha)	(resid 14 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 14 and name hn)	(resid 15 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 14 and name ha)	(resid 15 and name hn)	2.6 2.6 0.0	!sing_m
assign (resid 15 and name hn)	(resid 16 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 15 and name ha)	(resid 16 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 15 and name ha)	(resid 31 and name hg)	3.6 3.6 0.0	!sing_l
assign (resid 16 and name hn)	(resid 17 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 16 and name hn)	(resid 31 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 16 and name hn)	(resid 31 and name hg)	5.5 5.5 0.0	!sing_l
assign (resid 16 and name ha)	(resid 30 and name ha)	2.8 2.8 0.0	!sing_l
assign (resid 16 and name ha)	(resid 31 and name hn)	3.6 3.6 0.0	!sing_l
assign (resid 16 and name ha)	(resid 31 and name hg)	3.6 3.6 0.0	!sing_l
assign (resid 17 and name hn)	(resid 31 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 18 and name ha)	(resid 19 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 18 and name ha)	(resid 28 and name ha)	2.8 2.8 0.0	!sing_l
assign (resid 19 and name hn)	(resid 20 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 19 and name hn)	(resid 21 and name hd2)	5.5 5.5 0.0	!sing_m
assign (resid 19 and name hn)	(resid 28 and name ha)	5.5 5.5 0.0	!sing_l
assign (resid 19 and name ha)	(resid 20 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 19 and name hz)	(resid 60 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 20 and name ha)	(resid 21 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 20 and name ha)	(resid 26 and name ha)	3.6 3.6 0.0	!sing_l
assign (resid 21 and name hn)	(resid 21 and name hd2)	3.6 3.6 0.0	!sing_i
assign (resid 21 and name hn)	(resid 25 and name hn)	5.5 5.5 0.0	!sing_m
assign (resid 21 and name ha)	(resid 22 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 21 and name ha)	(resid 23 and name hn)	3.6 3.6 0.0	!sing_m
assign (resid 22 and name hn)	(resid 22 and name hg)	5.5 5.5 0.0	!sing_i
assign (resid 22 and name hn)	(resid 23 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 22 and name hn)	(resid 25 and name hn)	5.5 5.5 0.0	!sing_m
assign (resid 22 and name ha)	(resid 23 and name hn)	4.1 4.1 0.0	!sing_s
assign (resid 22 and name ha)	(resid 24 and name hn)	4.1 4.1 0.0	!sing_m
assign (resid 22 and name hg)	(resid 23 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 23 and name hn)	(resid 24 and name hn)	4.1 4.1 0.0	!sing_s
assign (resid 23 and name hn)	(resid 24 and name ha)	5.5 5.5 0.0	!sing_s
assign (resid 23 and name ha)	(resid 24 and name hn)	4.1 4.1 0.0	!sing_s
assign (resid 23 and name ha)	(resid 25 and name hn)	5.5 5.5 0.0	!sing_m
assign (resid 24 and name ha)	(resid 25 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 25 and name hn)	(resid 26 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 25 and name ha)	(resid 26 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 26 and name hn)	(resid 27 and name hn)	5.5 5.5 0.0	!sing_s

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assign (resid 26 and name ha)	(resid 27 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 27 and name ha)	(resid 28 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 28 and name ha)	(resid 29 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 29 and name ha)	(resid 30 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 30 and name hn)	(resid 31 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 30 and name ha)	(resid 31 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 31 and name hn)	(resid 31 and name hg)	3.6 3.6 0.0	!sing_i
assign (resid 31 and name hn)	(resid 32 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 31 and name ha)	(resid 32 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 31 and name ha)	(resid 31 and name hg)	3.6 3.6 0.0	!sing_i
assign (resid 32 and name ha)	(resid 32 and name hg)	3.6 3.6 0.0	!sing_i
assign (resid 32 and name ha)	(resid 33 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 32 and name ha)	(resid 34 and name hn)	3.6 3.6 0.0	!sing_m
assign (resid 33 and name hn)	(resid 34 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 33 and name ha)	(resid 34 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 34 and name hn)	(resid 35 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 34 and name hn)	(resid 44 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 34 and name ha)	(resid 35 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 34 and name ha)	(resid 43 and name ha)	2.8 2.8 0.0	!sing_l
assign (resid 34 and name ha)	(resid 44 and name hn)	3.1 3.1 0.0	!sing_l
assign (resid 35 and name hn)	(resid 36 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 35 and name hn)	(resid 41 and name ha)	5.5 5.5 0.0	!sing_l
assign (resid 35 and name hn)	(resid 42 and name hn)	3.6 3.6 0.0	!sing_l
assign (resid 35 and name hn)	(resid 43 and name ha)	5.5 5.5 0.0	!sing_l
assign (resid 35 and name hn)	(resid 44 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 35 and name hn)	(resid 44 and name ha)	5.5 5.5 0.0	!sing_l
assign (resid 35 and name ha)	(resid 36 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 36 and name hn)	(resid 37 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 36 and name ha)	(resid 37 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 36 and name ha)	(resid 41 and name ha)	2.8 2.8 0.0	!sing_l
assign (resid 36 and name ha)	(resid 42 and name hn)	3.6 3.6 0.0	!sing_l
assign (resid 37 and name hn)	(resid 40 and name hn)	3.6 3.6 0.0	!sing_m
assign (resid 37 and name hn)	(resid 41 and name ha)	3.5 3.6 0.0	!sing_m
assign (resid 37 and name hn)	(resid 42 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 37 and name ha)	(resid 38 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 38 and name hn)	(resid 39 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 39 and name hn)	(resid 40 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 40 and name hn)	(resid 41 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 40 and name ha)	(resid 41 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 41 and name hn)	(resid 42 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 41 and name ha)	(resid 42 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 42 and name ha)	(resid 43 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 42 and name hb)	(resid 43 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 43 and name hn)	(resid 44 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 43 and name ha)	(resid 44 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 44 and name ha)	(resid 46 and name hn)	3.6 3.6 0.0	!sing_m
assign (resid 45 and name ha)	(resid 46 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 46 and name ha)	(resid 72 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 47 and name ha)	(resid 48 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 48 and name hn)	(resid 49 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 48 and name hn)	(resid 51 and name hn)	5.5 5.5 0.0	!sing_m
assign (resid 48 and name ha)	(resid 49 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 48 and name ha)	(resid 50 and name hn)	5.5 5.5 0.0	!sing_m
assign (resid 48 and name hb)	(resid 49 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 48 and name hb)	(resid 50 and name hn)	5.5 5.5 0.0	!sing_m
assign (resid 49 and name hn)	(resid 50 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 49 and name hn)	(resid 51 and name hn)	5.5 5.5 0.0	!sing_m
assign (resid 49 and name ha)	(resid 50 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 49 and name ha)	(resid 51 and name hn)	4.1 4.1 0.0	!sing_m
assign (resid 49 and name ha)	(resid 53 and name ha)	3.6 3.6 0.0	!sing_m
assign (resid 49 and name ha)	(resid 54 and name hn)	3.6 3.6 0.0	!sing_l
assign (resid 50 and name hn)	(resid 51 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 51 and name hn)	(resid 52 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 51 and name hn)	(resid 53 and name hn)	4.1 4.1 0.0	!sing_m
assign (resid 51 and name hn)	(resid 54 and name hn)	4.1 4.1 0.0	!sing_m

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assign (resid 51 and name ha)	(resid 52 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 52 and name hn)	(resid 53 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 52 and name ha)	(resid 53 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 53 and name hn)	(resid 54 and name hn)	4.1 4.1 0.0	!sing_s
assign (resid 53 and name ha)	(resid 54 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 53 and name ha)	(resid 56 and name hn)	2.8 2.8 0.0	!sing_m
assign (resid 54 and name hn)	(resid 55 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 55 and name hn)	(resid 56 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 56 and name hn)	(resid 57 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 56 and name ha)	(resid 57 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 57 and name hn)	(resid 59 and name hn)	5.5 5.5 0.0	!sing_m
assign (resid 57 and name hn)	(resid 60 and name hn)	5.5 5.5 0.0	!sing_m
assign (resid 57 and name hn)	(resid 90 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 57 and name hn)	(resid 90 and name ha)	2.6 2.6 0.0	!sing_l
assign (resid 57 and name hn)	(resid 91 and name ha)	5.5 5.5 0.0	!sing_l
assign (resid 57 and name hn)	(resid 91 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 57 and name ha)	(resid 91 and name ha)	3.6 3.6 0.0	!sing_l
assign (resid 58 and name hn)	(resid 59 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 58 and name hn)	(resid 60 and name hn)	5.5 5.5 0.0	!sing_m
assign (resid 58 and name ha)	(resid 59 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 59 and name hn)	(resid 60 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 59 and name ha)	(resid 60 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 60 and name hn)	(resid 61 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 60 and name hn)	(resid 78 and name ha)	5.5 5.5 0.0	!sing_l
assign (resid 60 and name ha)	(resid 61 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 60 and name ha)	(resid 77 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 60 and name ha)	(resid 78 and name ha)	2.8 2.8 0.0	!sing_l
assign (resid 60 and name ha)	(resid 79 and name hn)	3.6 3.6 0.0	!sing_l
assign (resid 61 and name hn)	(resid 77 and name hn)	3.6 3.6 0.0	!sing_l
assign (resid 61 and name hn)	(resid 78 and name ha)	3.6 3.6 0.0	!sing_l
assign (resid 61 and name hn)	(resid 79 and name hn)	5.5 5.5 0.0	!sing_l
assign (resid 61 and name ha)	(resid 62 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 62 and name ha)	(resid 63 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 62 and name ha)	(resid 76 and name ha)	2.8 2.8 0.0	!sing_l
assign (resid 63 and name hn)	(resid 63 and name hb)	3.6 3.6 0.0	!sing_i
assign (resid 63 and name hn)	(resid 64 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 63 and name hn)	(resid 75 and name hn)	3.6 3.6 0.0	!sing_l
assign (resid 63 and name hn)	(resid 76 and name ha)	5.5 5.5 0.0	!sing_l
assign (resid 63 and name ha)	(resid 64 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 63 and name hb)	(resid 64 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 64 and name ha)	(resid 74 and name ha)	2.8 2.8 0.0	!sing_l
assign (resid 65 and name ha)	(resid 66 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 66 and name hn)	(resid 67 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 67 and name hn)	(resid 68 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 67 and name ha)	(resid 68 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 70 and name hn)	(resid 71 and name hn)	3.1 3.1 0.0	!sing_s
assign (resid 70 and name ha)	(resid 71 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 71 and name hn)	(resid 72 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 72 and name hn)	(resid 73 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 72 and name ha)	(resid 73 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 73 and name hn)	(resid 74 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 73 and name ha)	(resid 74 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 74 and name hn)	(resid 74 and name hb)	3.1 3.1 0.0	!sing_i
assign (resid 74 and name ha)	(resid 75 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 75 and name hn)	(resid 75 and name hb)	3.1 3.1 0.0	!sing_i
assign (resid 75 and name ha)	(resid 76 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 75 and name hb)	(resid 76 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 76 and name ha)	(resid 77 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 77 and name hn)	(resid 78 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 77 and name ha)	(resid 78 and name hn)	2.8 2.8 0.0	!sing_s
assign (resid 78 and name hn)	(resid 79 and name hn)	5.5 5.5 0.0	!sing_s
assign (resid 78 and name ha)	(resid 79 and name hn)	2.6 2.6 0.0	!sing_s
assign (resid 79 and name hn)	(resid 79 and name hb)	3.6 3.6 0.0	!sing_i
assign (resid 79 and name hn)	(resid 80 and name hn)	3.6 3.6 0.0	!sing_s
assign (resid 79 and name hb)	(resid 80 and name hn)	5.5 5.5 0.0	!sing_s

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assign (resid 81 and name ha) (resid 82 and name hn) 3.6 3.6 0.0 !sing_s
assign (resid 81 and name ha) (resid 83 and name hn) 4.1 4.1 0.0 !sing_m
assign (resid 82 and name hn) (resid 83 and name hn) 4.1 4.1 0.0 !sing_s
assign (resid 82 and name ha) (resid 83 and name hn) 4.1 4.1 0.0 !sing_s
assign (resid 83 and name hn) (resid 84 and name hn) 5.5 5.5 0.0 !sing_s
assign (resid 83 and name ha) (resid 84 and name hn) 2.6 2.6 0.0 !sing_s
assign (resid 84 and name hn) (resid 92 and name hn) 5.5 5.5 0.0 !sing_l
assign (resid 84 and name hn) (resid 93 and name hn) 5.5 5.5 0.0 !sing_l
assign (resid 85 and name ha) (resid 86 and name hn) 2.6 2.6 0.0 !sing_s
assign (resid 85 and name ha) (resid 92 and name ha) 2.8 2.8 0.0 !sing_l
assign (resid 86 and name hn) (resid 86 and name hg) 3.6 3.6 0.0 !sing_i
assign (resid 86 and name hn) (resid 87 and name hn) 5.5 5.5 0.0 !sing_s
assign (resid 86 and name hn) (resid 89 and name hn) 4.1 4.1 0.0 !sing_m
assign (resid 86 and name hn) (resid 90 and name hn) 5.5 5.5 0.0 !sing_m
assign (resid 86 and name hn) (resid 91 and name hn) 3.6 3.6 0.0 !sing_l
assign (resid 86 and name ha) (resid 87 and name hn) 3.6 3.6 0.0 !sing_s
assign (resid 87 and name hn) (resid 88 and name hn) 4.1 4.1 0.0 !sing_s
assign (resid 87 and name ha) (resid 88 and name hn) 3.1 3.1 0.0 !sing_s
assign (resid 88 and name hn) (resid 89 and name hn) 4.1 4.1 0.0 !sing_s
assign (resid 88 and name hn) (resid 90 and name hn) 5.5 5.5 0.0 !sing_m
assign (resid 88 and name ha) (resid 89 and name hn) 4.1 4.1 0.0 !sing_s
assign (resid 89 and name hn) (resid 90 and name hn) 3.6 3.6 0.0 !sing_s
assign (resid 90 and name hn) (resid 90 and name hb) 3.6 3.6 0.0 !sing_i
assign (resid 90 and name hn) (resid 91 and name hn) 3.1 3.1 0.0 !sing_s
assign (resid 90 and name hb) (resid 91 and name hn) 5.5 5.5 0.0 !sing_s
assign (resid 91 and name hn) (resid 92 and name hn) 5.5 5.5 0.0 !sing_s
assign (resid 91 and name ha) (resid 92 and name hn) 3.1 3.1 0.0 !sing_s
assign (resid 92 and name ha) (resid 93 and name hn) 3.1 3.1 0.0 !sing_s

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class hbnd

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assign (resid 17 and name hn) (resid 29 and name o) 2.0 0.3 0.3 !hbnd
assign (resid 17 and name n) (resid 29 and name o) 3.3 0.3 0.3 !hbnd
assign (resid 17 and name o) (resid 29 and name hn) 2.0 0.3 0.3 !hbnd
assign (resid 17 and name o) (resid 29 and name n) 3.3 0.3 0.3 !hbnd
assign (resid 19 and name hn) (resid 27 and name o) 2.0 0.3 0.3 !hbnd
assign (resid 19 and name n) (resid 27 and name o) 3.3 0.3 0.3 !hbnd
assign (resid 19 and name o) (resid 27 and name hn) 2.0 0.3 0.3 !hbnd
assign (resid 19 and name o) (resid 27 and name n) 3.3 0.3 0.3 !hbnd
assign (resid 35 and name hn) (resid 42 and name o) 2.0 0.3 0.3 !hbnd
assign (resid 35 and name n) (resid 42 and name o) 3.3 0.3 0.3 !hbnd
assign (resid 35 and name o) (resid 42 and name hn) 2.0 0.3 0.3 !hbnd
assign (resid 35 and name o) (resid 42 and name n) 3.3 0.3 0.3 !hbnd
assign (resid 61 and name hn) (resid 77 and name o) 2.0 0.3 0.3 !hbnd
assign (resid 61 and name n) (resid 77 and name o) 3.3 0.3 0.3 !hbnd
assign (resid 61 and name o) (resid 77 and name hn) 2.0 0.3 0.3 !hbnd
assign (resid 61 and name o) (resid 77 and name n) 3.3 0.3 0.3 !hbnd
assign (resid 63 and name hn) (resid 75 and name o) 2.0 0.3 0.3 !hbnd
assign (resid 63 and name n) (resid 75 and name o) 3.3 0.3 0.3 !hbnd
assign (resid 63 and name o) (resid 75 and name hn) 2.0 0.3 0.3 !hbnd
assign (resid 63 and name o) (resid 75 and name n) 3.3 0.3 0.3 !hbnd

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! dihedral angle restraints X-PLOR format

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!
! chi-1      22 restraints
! phi        25 restraints
! psi        33 restraints
! chi-2       5 restraints
!

```

!&lt;ENERGY&gt;

&lt;ANGLE&gt; &lt;RANGE&gt;&lt;EXPONENT&gt;

! chi-1 restraints

```

assign (resid 15 and name n ) (resid 15 and name ca)
      (resid 15 and name cb) (resid 15 and name cg) 1.0 -60.0 40.0 2

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```

assign (resid 19 and name n ) (resid 19 and name ca)
      (resid 19 and name cb) (resid 19 and name cg) 1.0 180.0 40.0 2
assign (resid 22 and name n ) (resid 22 and name ca)
      (resid 22 and name cb) (resid 22 and name cg) 1.0 -60.0 40.0 2
assign (resid 23 and name n ) (resid 23 and name ca)
      (resid 23 and name cb) (resid 23 and name cg) 1.0 60.0 40.0 2
assign (resid 30 and name n ) (resid 30 and name ca)
      (resid 30 and name cb) (resid 30 and name sg) 1.0 -60.0 40.0 2
assign (resid 31 and name n ) (resid 31 and name ca)
      (resid 31 and name cb) (resid 31 and name cg) 1.0 -60.0 40.0 2
assign (resid 32 and name n ) (resid 32 and name ca)
      (resid 32 and name cb) (resid 32 and name cg) 1.0 -60.0 40.0 2
assign (resid 33 and name n ) (resid 33 and name ca)
      (resid 33 and name cb) (resid 33 and name cg) 1.0 -60.0 40.0 2
assign (resid 34 and name n ) (resid 34 and name ca)
      (resid 34 and name cb) (resid 34 and name cg) 1.0 -60.0 40.0 2
assign (resid 35 and name n ) (resid 35 and name ca)
      (resid 35 and name cb) (resid 35 and name cg) 1.0 60.0 40.0 2
assign (resid 41 and name n ) (resid 41 and name ca)
      (resid 41 and name cb) (resid 41 and name sg) 1.0 -60.0 40.0 2
assign (resid 42 and name n ) (resid 42 and name ca)
      (resid 42 and name cb) (resid 42 and name cg1) 1.0 -60.0 40.0 2
assign (resid 49 and name n ) (resid 49 and name ca)
      (resid 49 and name cb) (resid 49 and name sg) 1.0 -60.0 40.0 2
assign (resid 52 and name n ) (resid 52 and name ca)
      (resid 52 and name cb) (resid 52 and name cg) 1.0 60.0 40.0 2
assign (resid 53 and name n ) (resid 53 and name ca)
      (resid 53 and name cb) (resid 53 and name cg) 1.0 180.0 40.0 2
assign (resid 56 and name n ) (resid 56 and name ca)
      (resid 56 and name cb) (resid 56 and name sg) 1.0 -60.0 40.0 2
assign (resid 62 and name n ) (resid 62 and name ca)
      (resid 62 and name cb) (resid 62 and name sg) 1.0 180.0 40.0 2
assign (resid 74 and name n ) (resid 74 and name ca)
      (resid 74 and name cb) (resid 74 and name cg1) 1.0 -60.0 40.0 2
assign (resid 76 and name n ) (resid 76 and name ca)
      (resid 76 and name cb) (resid 76 and name sg) 1.0 -60.0 40.0 2
assign (resid 78 and name n ) (resid 78 and name ca)
      (resid 78 and name cb) (resid 78 and name sg) 1.0 -60.0 40.0 2
assign (resid 87 and name n ) (resid 87 and name ca)
      (resid 87 and name cb) (resid 87 and name cg) 1.0 -60.0 40.0 2
assign (resid 92 and name n ) (resid 92 and name ca)
      (resid 92 and name cb) (resid 92 and name sg) 1.0 -60.0 40.0 2

```

**! phi restraints**

```

assign (resid 14 and name c ) (resid 15 and name n )
      (resid 15 and name ca) (resid 15 and name c ) 1.0 60.0 50.0 2 !HN(CO)HB
assign (resid 18 and name c ) (resid 19 and name n )
      (resid 19 and name ca) (resid 19 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.9 Hz
assign (resid 27 and name c ) (resid 28 and name n )
      (resid 28 and name ca) (resid 28 and name c ) 1.0 -120.0 70.0 2 !HNHA 7.6 Hz
assign (resid 29 and name c ) (resid 30 and name n )
      (resid 30 and name ca) (resid 30 and name c ) 1.0 -78.0 50.0 2
assign (resid 31 and name c ) (resid 32 and name n )
      (resid 32 and name ca) (resid 32 and name c ) 1.0 -84.0 50.0 2
assign (resid 32 and name c ) (resid 33 and name n )
      (resid 33 and name ca) (resid 33 and name c ) 1.0 60.0 50.0 2 !HN(CO)HB
assign (resid 33 and name c ) (resid 34 and name n )
      (resid 34 and name ca) (resid 34 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.2 Hz
assign (resid 34 and name c ) (resid 35 and name n )
      (resid 35 and name ca) (resid 35 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.45 Hz
assign (resid 36 and name c ) (resid 37 and name n )
      (resid 37 and name ca) (resid 37 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.6 Hz
assign (resid 38 and name c ) (resid 39 and name n )
      (resid 39 and name ca) (resid 39 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.6 Hz
assign (resid 39 and name c ) (resid 40 and name n )
      (resid 40 and name ca) (resid 40 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.3 Hz

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assign (resid 40 and name c ) (resid 41 and name n )
      (resid 41 and name ca) (resid 41 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.8 Hz
assign (resid 41 and name c ) (resid 42 and name n )
      (resid 42 and name ca) (resid 42 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.5 Hz
assign (resid 49 and name c ) (resid 50 and name n )
      (resid 50 and name ca) (resid 50 and name c ) 1.0 -75.0 50.0 2
assign (resid 50 and name c ) (resid 51 and name n )
      (resid 51 and name ca) (resid 51 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.5 Hz
assign (resid 52 and name c ) (resid 53 and name n )
      (resid 53 and name ca) (resid 53 and name c ) 1.0 60.0 50.0 2 !HN(CO)HB
assign (resid 55 and name c ) (resid 56 and name n )
      (resid 56 and name ca) (resid 56 and name c ) 1.0 -60.0 50.0 2 !HNHA 5.0 Hz
assign (resid 58 and name c ) (resid 59 and name n )
      (resid 59 and name ca) (resid 59 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.8 Hz
assign (resid 59 and name c ) (resid 60 and name n )
      (resid 60 and name ca) (resid 60 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.2 Hz
assign (resid 60 and name c ) (resid 61 and name n )
      (resid 61 and name ca) (resid 61 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.1 Hz
assign (resid 61 and name c ) (resid 62 and name n )
      (resid 62 and name ca) (resid 62 and name c ) 1.0 -150.0 70.0 2 !HNHA 7.9 Hz
assign (resid 62 and name c ) (resid 63 and name n )
      (resid 63 and name ca) (resid 63 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.7 Hz
assign (resid 73 and name c ) (resid 74 and name n )
      (resid 74 and name ca) (resid 74 and name c ) 1.0 -120.0 50.0 2
assign (resid 74 and name c ) (resid 75 and name n )
      (resid 75 and name ca) (resid 75 and name c ) 1.0 -120.0 50.0 2 !HNHA 9.15 Hz
assign (resid 76 and name c ) (resid 77 and name n )
      (resid 77 and name ca) (resid 77 and name c ) 1.0 -120.0 50.0 2 !HNHA 8.2 Hz

```

## ! psi restraints

```

assign (resid 15 and name n ) (resid 15 and name ca)
      (resid 15 and name c ) (resid 16 and name n ) 1.0 50.0 50.0 2
assign (resid 19 and name n ) (resid 19 and name ca)
      (resid 19 and name c ) (resid 20 and name n ) 1.0 120.0 75.0 2
assign (resid 25 and name n ) (resid 25 and name ca)
      (resid 25 and name c ) (resid 26 and name n ) 1.0 120.0 75.0 2
assign (resid 27 and name n ) (resid 27 and name ca)
      (resid 27 and name c ) (resid 28 and name n ) 1.0 120.0 75.0 2
assign (resid 28 and name n ) (resid 28 and name ca)
      (resid 28 and name c ) (resid 29 and name n ) 1.0 120.0 75.0 2
assign (resid 29 and name n ) (resid 29 and name ca)
      (resid 29 and name c ) (resid 30 and name n ) 1.0 120.0 60.0 2
assign (resid 30 and name n ) (resid 30 and name ca)
      (resid 30 and name c ) (resid 31 and name n ) 1.0 125.0 75.0 2
assign (resid 31 and name n ) (resid 31 and name ca)
      (resid 31 and name c ) (resid 32 and name n ) 1.0 120.0 75.0 2
assign (resid 32 and name n ) (resid 32 and name ca)
      (resid 32 and name c ) (resid 33 and name n ) 1.0 174.0 50.0 2
assign (resid 33 and name n ) (resid 33 and name ca)
      (resid 33 and name c ) (resid 34 and name n ) 1.0 60.0 50.0 2
assign (resid 34 and name n ) (resid 34 and name ca)
      (resid 34 and name c ) (resid 35 and name n ) 1.0 159.0 50.0 2
assign (resid 35 and name n ) (resid 35 and name ca)
      (resid 35 and name c ) (resid 36 and name n ) 1.0 156.0 50.0 2
assign (resid 36 and name n ) (resid 36 and name ca)
      (resid 36 and name c ) (resid 37 and name n ) 1.0 120.0 60.0 2
assign (resid 37 and name n ) (resid 37 and name ca)
      (resid 37 and name c ) (resid 38 and name n ) 1.0 120.0 60.0 2
assign (resid 40 and name n ) (resid 40 and name ca)
      (resid 40 and name c ) (resid 41 and name n ) 1.0 120.0 60.0 2
assign (resid 41 and name n ) (resid 41 and name ca)
      (resid 41 and name c ) (resid 42 and name n ) 1.0 120.0 60.0 2
assign (resid 42 and name n ) (resid 42 and name ca)
      (resid 42 and name c ) (resid 43 and name n ) 1.0 120.0 60.0 2
assign (resid 43 and name n ) (resid 43 and name ca)
      (resid 43 and name c ) (resid 44 and name n ) 1.0 120.0 60.0 2
assign (resid 45 and name n ) (resid 45 and name ca)

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assign (resid 45 and name c ) (resid 46 and name n ) 1.0 -60.0 50.0 2 !HNHB
(resid 51 and name n ) (resid 51 and name ca)
assign (resid 51 and name c ) (resid 52 and name n ) 1.0 120.0 75.0 2
(resid 53 and name n ) (resid 53 and name ca)
assign (resid 53 and name c ) (resid 54 and name n ) 1.0 50.0 50.0 2
(resid 58 and name n ) (resid 58 and name ca)
assign (resid 58 and name c ) (resid 59 and name n ) 1.0 -60.0 50.0 2 !HNHB
(resid 59 and name n ) (resid 59 and name ca)
assign (resid 59 and name c ) (resid 60 and name n ) 1.0 33.0 50.0 2
(resid 60 and name n ) (resid 60 and name ca)
assign (resid 60 and name c ) (resid 61 and name n ) 1.0 162.0 50.0 2
(resid 61 and name n ) (resid 61 and name ca)
assign (resid 61 and name c ) (resid 62 and name n ) 1.0 120.0 60.0 2
(resid 62 and name n ) (resid 62 and name ca)
assign (resid 62 and name c ) (resid 63 and name n ) 1.0 120.0 60.0 2
(resid 63 and name n ) (resid 63 and name ca)
assign (resid 63 and name c ) (resid 64 and name n ) 1.0 165.0 50.0 2
(resid 74 and name n ) (resid 74 and name ca)
assign (resid 74 and name c ) (resid 75 and name n ) 1.0 120.0 60.0 2
(resid 75 and name n ) (resid 75 and name ca)
assign (resid 75 and name c ) (resid 76 and name n ) 1.0 120.0 60.0 2
(resid 76 and name n ) (resid 76 and name ca)
assign (resid 76 and name c ) (resid 77 and name n ) 1.0 120.0 60.0 2
(resid 78 and name n ) (resid 78 and name ca)
assign (resid 78 and name c ) (resid 79 and name n ) 1.0 120.0 60.0 2
(resid 83 and name n ) (resid 83 and name ca)
assign (resid 83 and name c ) (resid 84 and name n ) 1.0 120.0 60.0 2
(resid 92 and name n ) (resid 92 and name ca)
assign (resid 92 and name c ) (resid 93 and name n ) 1.0 120.0 75.0 2

```

## ! chi-2 restraints

```

assign (resid 2 and name ca) (resid 2 and name cb)
(resid 2 and name cgl) (resid 2 and name cd1) 1.0 -60.0 40.0 2 !LRCH
assign (resid 31 and name ca) (resid 31 and name cb)
(resid 31 and name cg) (resid 31 and name cd1) 1.0 180.0 40.0 2 !LRCH
assign (resid 32 and name ca) (resid 32 and name cb)
(resid 32 and name cg) (resid 32 and name cd1) 1.0 180.0 40.0 2 !LRCH
assign (resid 74 and name ca) (resid 74 and name cb)
(resid 74 and name cgl) (resid 74 and name cd1) 1.0 -60.0 40.0 2 !LRCH
assign (resid 90 and name ca) (resid 90 and name cb)
(resid 90 and name cgl) (resid 90 and name cd1) 1.0 180.0 40.0 2 !LRCH

```

**Example 3: Identification of blocking antibodies using a competitive binding assay and immobilised wild type GST-MSP-1<sub>19</sub>.**

In previous studies antibodies that blocked the action of the neutralising antibodies 12.8 and 12.10 had been defined either directly in the MSP-1<sub>42</sub> processing assay (Blackman *et al.*, 1994) in a coupled erythrocyte invasion-MSP-1<sub>42</sub> processing assay (Guevara *et al.*, 1997) or in a competitive radioimmunoassay with merozoite protein as the antigen (Guevara *et al.*, 1997). These studies have been extended using recombinant MSP-1 and BIAcore analysis.

A recombinant fusion protein comprising wild type MSP-1<sub>19</sub> fused to GST was coupled to the sensor chip and competitor antibody was first allowed to bind to the antigen. Then a solution of either mAb 12.8 or 12.10 was passed over the chip and the amount of binding of this second antibody was quantified. If the first antibody interferes with the binding of the second antibody then this is reflected in a reduction in the amount of second antibody bound.

Methods

The wild type GST-MSP-1<sub>19</sub> was coupled to a CM5 sensor chip. The binding assays were performed with a constant flow rate of 5  $\mu\text{l min}^{-1}$  at 25°C. For binding, purified mAbs 1E1, 8A12 and 2F10 at 100  $\mu\text{g ml}^{-1}$  in HBS-EP buffer (10mM HEPES pH7.4 containing 150mM NaCl, 3mM EDTA and 0.005%v/v polysorbate 20); mAbs 1E8, 9C8, 12D11, 111.2 and 111.4 in cell culture medium supernatant; mAbs 2.2, 7.5 and 89.1 at 1:10 dilution of ascitic fluid in HBS-EP buffer; and mouse  $\alpha$ -GST antibody at 1:10 dilution serum in HBS-EP buffer were allowed to interact with immobilised wild type GST-MSP-1<sub>19</sub> for 10 min. After allowing 5 min for dissociation of low affinity interactions, either mAb 12.8 or 12.10 at 100  $\mu\text{g ml}^{-1}$  in HBS-EP buffer was added and allowed to bind for 10 min. After washing the chip for 5 min the binding of 12.8 or 12.10 was measured. The chip was regenerated by washing off bound antibody with 10 mM glycine-HCl, pH 2.4, or when required with 100 mM glycine-HCl, pH 1.8, for 3 min.

## Results

The results are shown in Figure 12. All the competitor antibodies bind to the GST-MSP-1<sub>19</sub> antigen, with the exception of mAb89.1 which is a negative control. As expected, mAbs 12.8 and 12.10 competed with each other (Guevara *et al.*, 1997). The other antibodies which do not inhibit processing could to greater or lesser interfere with the binding of 12.8 and 12.10. As expected from previous studies mAbs 1E1, and 7.5 blocked both 12.8 and 12.10, whereas 2.2 and 11.4 blocked 12.8. Another particularly effective blocking antibody identified in this study was mAb9C8.

### **Example 4: Immunization of small animals with modified GST-MSP-1<sub>19</sub> and analysis of the antibodies induced**

To determine whether or not the modified proteins were immunogenic, recombinant GST-MSP-1<sub>19</sub> fusion proteins were used to raise antibodies by immunisation.

## Methods

Two modified proteins containing either 3 [27+31+43] or 4 [15+27+31+43] amino acid substitutions, respectively, were used to immunise rabbits and mice. The rabbits were immunised subcutaneously with MSP-1<sub>19</sub> protein in Freund's complete adjuvant and then boosted on three occasions with 200 µg of the protein in Freund's incomplete adjuvant 21, 42 and 63 days later, and serum samples were collected.

The presence and level of antibodies binding to the native MSP-1 protein in the parasite was assessed by indirect immunofluorescence using acetone fixed smears of parasite-infected erythrocytes. The sera were diluted serially in phosphate buffered saline (PBS) and incubated on the slide for 30 min at room temperature. After washing, the slides were incubated with FITC conjugated goat anti-rabbit or anti-mouse IgG, washed, and then examined by fluorescence microscopy.

The sera were also analysed in an MSP-1 secondary processing assay. Analysis and quantitation of secondary processing of MSP-1 in merozoite preparations was by a

modification of an assay described previously (Blackman *et al.*, 1994). Washed *P. falciparum* 3D7 merozoites were resuspended in ice-cold 50 mM Tris-HCl pH 7.5 containing 10 mM CaCl<sub>2</sub> and 2 mM MgCl<sub>2</sub> (reaction buffer). Aliquots of about  $1 \times 10^9$  merozoites were dispensed into 1.5 ml centrifuge tubes on ice, and the parasites pelleted  
5 in a microfuge at 13,000 x g for 2 minutes at 4°C. The supernatant was removed, and individual merozoite pellets were then resuspended on ice in 25 µl of reaction buffer further supplemented with protease inhibitor or antibodies as appropriate. Merozoites were maintained on ice for 20 min to allow antibody binding, then transferred to a 37°C water bath for one hour to allow processing to proceed. Assays always included the  
10 following controls: a "positive processing" control sample of merozoites resuspended in reaction buffer only; a "negative processing" control sample of merozoites resuspended in reaction buffer plus 1mM PMSF; and a zero time (0h) control, in which processing was stopped before the 37°C incubation step. The processing was assayed using the western blot-based method and by a modified processing assay. Supernatants from the assays  
15 were obtained after centrifugation for 30 min at 4°C, 13,000 x g to remove the insoluble material. The amount of MSP-1<sub>33</sub> in the supernatants was measured using an ELISA method. Fifty microlitres of diluted sample supernatants were added to the wells of an ELISA plate (NUNC F96 Cert. Maxisorp) that had been coated with 100 µl/well of 4 µg ml<sup>-1</sup> human mAb X509 in PBS. Plates were incubated for 4 hours at 37°C and then  
20 washed 3 times with 0.01% PBS-Tween (PBS-T). Bound MSP-1<sub>33</sub> protein was detected by addition of 100 µl of 1:4000 dilution of mouse mAb G13 for 1 hour at 37°C, followed by washing and the addition of 100 µl of 1:1000 dilution of sheep anti-mouse IgG (H+L) HRP-conjugated antibody. After incubation for 1 h at 37°C, the plates were washed again and HRP was detected by the addition of 100 µl of freshly prepared substrate solution  
25 (400mg l<sup>-1</sup> o-phenylenediamine dihydrochloride in 0.05 M phosphate buffer, 0.024 M citric acid and 0.012% H<sub>2</sub>O<sub>2</sub>) at room temperature for 20 min. The reaction was stopped by adding 10 µl of 1 M sulphuric acid and the absorbance of each sample was measured at 492 nm.

### 30 Results

The results are shown in Figure 13. The two modified proteins produced antibodies that reacted with MSP-1 in the parasite-infected erythrocyte, with a serum titre of 1:10,000,

which was an identical titre to that of a serum produced in the same way by immunisation with a recombinant protein containing the wildtype MSP-1 sequence. This indicates that the modified proteins can produce antibodies that react with the native protein. The antibodies induced by immunization were able to partially inhibit processing at the concentration used in a preliminary experiment, whereas in the control serum no antibodies that inhibited processing were present.

**Example 5: Design and Synthesis of a *Plasmodium Falciparum* Merozoite Surface Protein-1 Gene Fragment Optimized for *Pichia Pastoris* Heterologous Expression**

The coding sequence of the *Plasmodium falciparum* merozoite surface protein-1 (MSP1) 41.1 kDa processed fragment (MSP-142) has been redesigned for optimal heterologous expression in the yeast *Pichia pastoris*. The optimized DNA sequence was synthesized by PCR gene assembly, in the form of two fragments, MSP-133 and MSP-119. *P. pastoris* was transformed with an expression vector containing the optimized MSP-119 construct. Recombinant strains were shown to express high levels of non-glycosylated, properly folded MSP-119 protein.

Proteins encoded by the AT-rich genome of the human malaria parasite *Plasmodium falciparum* are generally poorly expressed in heterologous systems (Withers-Martinez et al., 1999). The methylotrophic yeast *Pichia (Komagataella) pastoris* is an appropriate system for expression of disulphide-bridged proteins such as the C-terminal fragment of the *P. falciparum* merozoite surface protein-1 (MSP1) (White et al., 1994; Morgan et al., 1999). In the *P. pastoris* expression system, it is important to avoid premature transcription termination due to AT-rich stretches (Romanos et al., 1991). Codon preferences for highly expressed genes in *P. pastoris* have been identified (Sreekrishna et al., EP 0 586 892 A1). Therefore, a synthetic MSP-142 gene fragment with codon usage optimized for *P. pastoris* expression was designed, using novel computer software (Withers-Martinez et al., 1999). It has previously been shown that the MSP-119 fragment is partially glycosylated when expressed in *P. pastoris*, and the carbohydrate must be enzymatically removed during purification (Morgan et al., 1999). Therefore, two specific point mutations were introduced into the synthetic MSP-142 protein sequence in order to

prevent N-linked glycosylation at NxS/T sites (one potential site within the MSP-133 sequence, and a known site within the MSP-119 sequence at Asn 1).

The optimized MSP-142 sequence was synthesized by gene assembly polymerase chain  
5 reaction (Stemmer et al., 1995, Withers-Martinez et al., 1999), in the form of separate  
MSP-133 and MSP-119 fragments. The optimized MSP-119 fragment was subcloned into  
a novel modified *Pichia* expression vector, transformed into the *P. pastoris* host strain  
SMD1168, and several independent transformants were isolated. These transformants  
were shown to efficiently express non-glycosylated, properly folded MSP-119. Strong  
10 expression of the optimized gene was observed in low copy number transformants. A  
multiple copy transformant with intermediate level G418 resistance gave expression of  
purified MSP-119 at a level equal to the high-expressing strain previously described  
(Morgan et al., 1999), which contains the original *P. falciparum* DNA. Thus, it should be  
possible to obtain even higher yields from high level G418-resistant transformants of the  
15 synthetic optimized gene.

#### Methods: Gene Assembly

The *P. falciparum* MSP-142 (41.1 kDa) fragment protein sequence SWISS-PROT  
20 accession number P04933: positions 1264-1621) was first altered to eliminate N-linked  
glycosylation signals by 2 amino acid substitutions. The sequences NYT (in the N-  
terminal portion; position 1445) and NIS (at the beginning of the C-terminal fragment;  
position 1526) were changed to QYT and NIA respectively. The protein sequence was  
then reverse-translated with DNA-STAR using the *S. cerevisiae* codon preferences. This  
25 sequence was used as input for the CODOP program (Withers-Martinez et al., 1999). Ten  
random sequences were generated with this program, using a codon weighting table  
(Figure 14) derived from codon usage in highly expressed *P. pastoris* genes (Sreekrishna  
et al., EP 0 586 892 A1). Thus, the codon table should reflect usage in highly expressed  
genes, rather than average usage. The random sequence that contained the minimum  
30 number of unfavourable codons (6) was selected, and these codons were changed  
manually to more preferred alternatives. The sequence was then analysed with DNA-  
STAR to check for AT-rich sequences that may cause transcription termination, and for

direct and inverted repeats. A set of 50 overlapping oligonucleotides coding for the final sequence was then generated. This consisted of 49 oligonucleotides of length 42 nt, and one of length 48 nt. Each oligonucleotide had a 21 bp overlap with its neighbours, with no gaps. Estimated  $T_m$ s were in the range of 60°C to 77°C. Oligonucleotides were synthesised  
5 by Oswel (Southampton, UK) at 40 nmol scale, and supplied in deionised water without purification. Outside primers of various lengths for the amplification step were also synthesised, to give a  $T_m$  of 62°C to 64°C, and contained a 5'-terminal phosphate group for ligation following the amplification step. The reverse primers also included a translation termination codon (UAA in the complementary strand). All oligonucleotides  
10 were diluted to 10  $\mu$ M in ddH<sub>2</sub>O before use.

The PCR-mediated gene assembly and amplification were carried out as described (Stemmer *et al.*, 1995; Withers-Martinez *et al.*, 1999), using a Biometra cyclor, in thin-walled 200  $\mu$ L tubes, under the following conditions.

15

Gene assembly reactions (Reaction 1):

50  $\mu$ L volume

2 units Vent polymerase (New England Biolabs)  
20 0.4 mM dNTPs  
1 x Vent polymerase buffer  
Oligonucleotide mix containing each oligonucleotide at 200 nM

Cycles:

25 32 cycles (2 h 33 m)  
denaturation 94°C 30 s  
annealing 52°C 30 s  
extension 72°C 3 m

30 Three fragments of the MSP-142 (41.1 kDa) region were synthesised separately with different outside primers and subsets of the 50 oligonucleotide set:

N-terminal fragment (bp 1-423) 21 oligonucleotides

middle fragment (bp 337-786) 22 oligonucleotides

C-terminal fragment (bp 787-1074) 14 oligonucleotides

- 5 The C-terminal fragment produces a 10.6 kDa fragment (MSP-119). The N-terminal and middle fragments, which overlap between positions 337 and 423, were subsequently spliced together at the BglII site (371-376) to give a 786 bp fragment that encodes the 30.5 kDa MSP-133 protein.

10 Amplification reactions (Reaction 2):

100  $\mu$ L volume

10  $\mu$ L aliquot of the gene assembly reaction

4 units Vent polymerase

15 0.4 mM dNTPs

1 x Vent polymerase buffer

1  $\mu$ M outside primers

Cycles:

20 32 cycles (2 h 55 m)

denaturation 94°C 45 s

annealing 52°C 45 s

extension 72°C 3 m

final extension 72°C 5 m

25

The PCR products were then purified by filtration with Centricon-100 units (Amicon), and cloned directly into the vectors by blunt-end ligation overnight at 16°C with T4 DNA ligase.

- 30 The synthetic MSP-119 gene was cloned directly into a *P. pastoris* expression vector. The modified pPIC9KHXa vector, containing a His<sub>6</sub> tag and factor Xa cleavage site (see Figure 15) inserted in the pPIC9K SnaBI site, had been digested with PmlI and treated



with calf alkaline phosphatase. The HXa vector had been previously created by insertion of a 36 bp synthetic oligonucleotide, containing the His<sub>6</sub> tag, factor Xa cleavage site, and PmlI restriction site into the SnaBI site of the pPIC9K vector.

5 The N-terminal and middle fragment PCR products were cloned into the SmaI site of the dephosphorylated pUC118 vector. Plasmid clones containing inserts were sequenced. Clones with the correct synthetic sequence were then digested and the two fragments were gel-purified. The N-terminal fragment clones were digested with EcoRI and BglII, and the middle fragment clones were digested with HindIII and BglII. The recombinant fragments  
10 were purified on an agarose gel, and eluted with a QIAGEN extraction kit. The purified N-terminal and middle fragments were then spliced together by ligation into a pUC118 vector that had been digested with HindIII and EcoRI and treated with calf alkaline phosphatase. This created the complete synthetic MSP-133 coding sequence. The N-terminal and middle fragment PCR products were cloned into the SmaI site of the  
15 dephosphorylated pUC118 vector. Plasmid clones containing inserts were sequenced. Clones with the correct synthetic sequence were then digested and the two fragments were gel-purified. The N-terminal fragment clones were digested with EcoRI and BglII, and the middle fragment clones were digested with HindIII and BglII. The recombinant fragments were purified on an agarose gel, and eluted with a QIAGEN extraction kit. The purified  
20 N-terminal and middle fragments were then spliced together by ligation into a pUC118 vector that had been digested with HindIII and EcoRI and treated with calf alkaline phosphatase. This created the complete synthetic MSP-133 coding sequence.

#### Methods: Expression and Purification

25

The methylotrophic yeast *Pichia* (*Komagataella*) *pastoris* strain SMD1168 was transformed by electroporation as described previously (Morgan et al., 1999). In addition, some G418-resistant clones were isolated using Hybond-N+ membranes (Fairlie et al., 1999).

30

Expression screening of transformants was performed by growing 10 ml cultures in buffered minimal glucose medium. Cells were harvested and resuspended in 10 ml

buffered minimal methanol medium at 1.0 OD<sub>600</sub> and grown overnight to a final OD<sub>600</sub> of 2.5 to 3.0. Cells were removed by centrifugation, and 1.2 ml of the supernatant medium was precipitated 30 min on ice with 15 % trichloroacetic acid. The samples were centrifuged for 30 min at 14000 rpm at 4 °C in a microfuge, and the protein pellets were washed twice with cold acetone. Samples were resuspended in 12 µl ddH<sub>2</sub>O, and 5 µl was electrophoresed, after reduction with DTT, on NOVEX pre-poured acrylamide gels according to manufacturer's instructions. NOVEX 4-12 % acrylamide gradient, or 10 % acrylamide, Bis/Tris gels in MES buffer were used. Protein gels were stained with Coomassie colloidal Brilliant Blue stain (Sigma).

Homogeneously purified MSP-119 was obtained as described previously (Morgan et al., 1999), except that enzymatic deglycosylation was omitted for the synthetic gene products.

#### Methods: NMR

One-dimensional <sup>1</sup>H- and 2-dimensional {<sup>1</sup>H/<sup>15</sup>N}-HSQC spectra were acquired as described previously (Morgan et al., 1999), at 25 °C, at sample concentrations of 1.1-2.5 mM.

#### Results

The sequences of the synthetic DNA fragments, and the resulting predicted protein products, are shown in Figure 15. A summary of the resulting improvements to the sequence is given in Table 3.

	Total codons	<i>P. pastoris</i> preferred codons	Unfavourable codons	% AT conte
<i>P. falciparum</i> MSP1 358 41.1 kDa fragment		140	28	74
Synthetic 41.1 kDa 358 fragment		276	0	58

Table 3. Codon usage

PCR-gene assembly reactions for the MSP-133 (two sections) and MSP-119 synthetic fragments are shown on agarose gels in Figure 16. This demonstrated that a single, correct size major product was observed in each case. The PCR products were subcloned, screened, and sequenced as described in the Methods section.

*P. pastoris* was transformed with the synthetic MSP-119 construct in the modified pPIC9K expression vector (pPIC9K-HXa; Figure 15). Expression of the synthetic MSP-119 product in three independent transformants is shown on a protein gel in Figure 17. The protein samples were prepared by trichloroacetic acid precipitation from culture supernatants as described in the Methods section. This demonstrated that a single, major product was present in each sample, corresponding to the expected migration of the synthetic MSP-119 protein. This migrated slightly more slowly than the control sample, which as described previously (Morgan et al., 1999) has a shorter N-terminal tag sequence. There was no trace of heterogeneous, slowly migrating recombinant protein that would result from glycosylation. Therefore, non-glycosylated, synthetic MSP-119 is efficiently expressed by the transformed yeast. The yield (measured by UV absorbance) of purified MSP-119 was 16 mg/L for low copy number transformants (resistant to 0.25 mg/ml G418), and increased to 24 mg/L for intermediate G418 resistance (resistant to 1.0 mg/ml G418). This can be compared with yields of 1-2 mg/L for low copy number transformants of *P. pastoris* with the original *Plasmodium falciparum* coding sequence,

before isolation of a highly G418-resistant strain (Morgan et al., 1999). This indicated that the synthetic MSP-119 construct is advantageous for recombinant protein expression, and that further improvement would result from isolation of higher copy number transformants.

5

One-dimensional proton NMR experiments demonstrated that the synthetic MSP-119 protein spectrum was very similar to the previously studied protein (Morgan et al., 1999), and represented a correctly folded protein (data not shown). This was further confirmed by a 2D- $\{^1\text{H}/^{15}\text{N}\}$ -HSQC spectra (Figure 18), which also shows that the structure of the  
10 synthetic product is identical to the previously studied protein, except for slight differences at the N-terminus which are consistent with the presence of a distinct N-terminal tag sequence, and S3->A mutation at the glycosylation site. Backbone NH proton and  $^{15}\text{N}$  chemical shifts for the original *P. falciparum* sequence product have been previously presented (Morgan et al., 1999). The similarity between the two spectra,  
15 outside of the N-terminal region, is strong evidence that both protein forms are in a structurally similar, correctly folded state.

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## CLAIMS

1. A non-naturally occurring variant of a C-terminal fragment of a *Plasmodium* merozoite surface protein-1 (MSP-1) wherein said variant has (i) a reduced affinity,  
5 compared with a naturally occurring *Plasmodium* MSP-1<sub>19</sub>, for at least one first antibody capable of blocking the binding of a second antibody, which second antibody inhibits the proteolytic cleavage of *Plasmodium* MSP-1<sub>42</sub> and (ii) substantially the same affinity for at least one third antibody compared with said naturally occurring *Plasmodium* MSP-1<sub>19</sub>, which third antibody inhibits the proteolytic cleavage of *Plasmodium* MSP-1<sub>42</sub>.
- 10 2. A variant according to claim 1 wherein said *Plasmodium* MSP-1<sub>19</sub> and MSP-1<sub>42</sub> are *Plasmodium falciparum* MSP-1<sub>19</sub> and MSP-1<sub>42</sub>.
3. A variant according to claim 1 or 2 wherein the first antibody is selected from  
15 mAbs 1E1, 2.2, 7.5, 9C8 and 111.4.
4. A variant according to claim 1 or 2 wherein the second and/or third antibody is selected from mAbs 12.8, 12.10 and 5B1.
- 20 5. A non-naturally occurring variant of a C-terminal fragment of a *Plasmodium* merozoite surface protein-1 (MSP-1) comprising an amino acid modification at any one of amino acid residues 14, 15, 27, 31, 34, 43, 48 and 53 of the *Plasmodium falciparum* MSP-1<sub>19</sub> amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.
- 25 6. A variant according to claim 5 wherein said modifications are selected from Gln14→Arg, Gln14→Gly, Asn15→Arg, Glu27→Tyr, Leu31→Arg, Tyr34→Ser, Tyr34→Ile, Glu43→Leu, Thr48→Lys and Asn53→Arg and their equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.
- 30 7. A variant according to claim 6 wherein said substitutions are selected from [Glu27→Tyr, Leu31→Arg and Glu43→Leu], [Glu27→Tyr, Leu31→Arg, Tyr34→Ser and Glu43→Leu], [Asn15→Arg, Glu27→Tyr, Leu31→Arg and Glu43→Leu] and their

equivalents in other *Plasmodium* MSP-1<sub>19</sub> polypeptides.

8. A variant according to any one of claims 5 to 7 further comprising a mutation at Cys12 and/or Cys28 of the *Plasmodium falciparum* MSP-1<sub>19</sub> amino acid sequence shown  
5 as SEQ I.D. No. 1.

9. A variant according to claim 8, in which the mutation is a substitution selected from Cys12→Ile and Cys28→Trp, and Cys12→Ala and Cys28→Phe.

10. A variant according to claim 9, in which the substitutions are selected from [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Glu43→Leu], [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Glu43→Leu, Asn53→Arg], [Cys12→Ile, Asn 15→Arg, Glu27→Tyr, Cys28→Trp, Leu31→Arg, Tyr34→Ser, Glu43→Leu, Asn53→Arg] and their equivalents in other *Plasmodium* MSP-  
15 1<sub>19</sub> polypeptides.

11. A method for producing a *Plasmodium* MSP-1 variant for use in preparing a vaccine composition which method comprises modifying one or more amino acid residues of a *Plasmodium* MSP-1 C-terminal fragment such that the resulting derivative has (i) a  
20 reduced affinity, compared with a naturally occurring *Plasmodium* MSP-1<sub>19</sub>, for at least one first antibody capable of blocking the binding of a second antibody, which second antibody inhibits the proteolytic cleavage of *Plasmodium* MSP-1<sub>42</sub> and (ii) substantially the same affinity for at least one third antibody compared with said naturally occurring *Plasmodium* MSP-1<sub>19</sub>, which third antibody inhibits the proteolytic cleavage of  
25 *Plasmodium* MSP-1<sub>42</sub>.

12. A non-naturally occurring *Plasmodium* MSP-1 variant obtained by the method of claim 11.

30 13. A polynucleotide encoding a variant according to any one of claims 1 to 10 or 12 operably linked to a regulatory sequence capable of directing the expression of said nucleotide in a host cell.

14. 14. A polynucleotide according to claim 13 having a sequence optimised for expression in said host cell.
15. A polynucleotide according to claim 13 or 14, in which the host cell is a *Pichia*  
5 *pastoris* cell.
16. A nucleic acid vector comprising a polynucleotide according to claim 13, 14 or 15.
17. A host cell comprising a vector according to claim 16.
- 10 18. A pharmaceutical composition comprising a variant according to any one of claims 1 to 10 or 12, a polynucleotide according to any one of claims 13 to 15 or a vector according to claim 16 together with a pharmaceutically acceptable carrier or diluent.
- 15 19. A composition according to claim 18 further comprising an immunogenic *Plasmodium* polypeptide or fragment or derivative thereof.
- 20 20. A method for producing anti-MSP-1 antibodies which method comprises administering a polypeptide according to any one of claims 1 to 10 or 12, a polynucleotide according to any one of claims 13 to 15 or a vector according to claim 16 to a mammal.
- 25 21. A method for producing polyclonal anti-MSP-1 antibodies which method comprises administering a polypeptide according to any one of claims 1 to 10 or 12, a polynucleotide according to any one of claims 13 to 15 or a vector according to claim 16 to a mammal and extracting the serum from said mammal.
22. An antibody produced by the method of claim 20 or 21.
- 30 23. A method of inducing immunity against malaria induced by *Plasmodium falciparum* which comprises administering to a person in need of such immunity an effective amount of the variant of claim 1, the polynucleotide of claim 13 or the vector of claim 16.

24. A method of immunizing a mammal, said method comprising administering an effective amount the polypeptide of claim 1, the polynucleotide of claim 13 or the vector of claim 16.
- 5 25. The method of claim 24, wherein said mammal is immunized against malaria.
26. A method of treating a malaria infection in a human patient which comprises administering to the patient an effective amount of the pharmaceutical composition of claim 18 or 19.
- 10 27. A variant according to any one of claims 1 to 10 or 12, a polynucleotide according to any one of claims 13 to 15 or a vector according to claim 16 for use in therapy.
- 15 28. A computer readable medium having stored thereon a model of the MSP-1<sub>19</sub> NMR structure.
29. A nucleic acid encoding a *Plasmodium* MSP-1 polypeptide, in which the nucleic acid is optimised for expression in a heterologous host cell.
- 20 30. A nucleic acid according to Claim 29, in which the heterologous host is a *Pichia pastoris* cell.
31. A nucleic acid according to Claim 29 or 30, in which the polypeptide is selected from an MSP-1<sub>42</sub> polypeptide comprising a sequence shown in Figures 2C and 2E, an MSP-1<sub>19</sub> polypeptide comprising a sequence shown in Figure 2C, and an MSP-1<sub>33</sub> polypeptide comprising a sequence shown in Figure 2E.
- 25 32. A nucleic acid according to any of Claims 29 to 31, in which the nucleic acid comprises a sequence selected from the sequences of Figure 2A, Figure 2B and Figure 2D.
- 30

33. A nucleic acid vector comprising a nucleic acid according to any of Claims 29 to

5 34. A host cell comprising a vector according to claim 33.

35. A pharmaceutical composition comprising a nucleic acid according to any of Claims 29 to 31 or a vector according to Claim 33 together with a pharmaceutically acceptable carrier or diluent.

10

36. A composition according to Claim 35 further comprising an immunogenic *Plasmodium* polypeptide or fragment or derivative thereof.

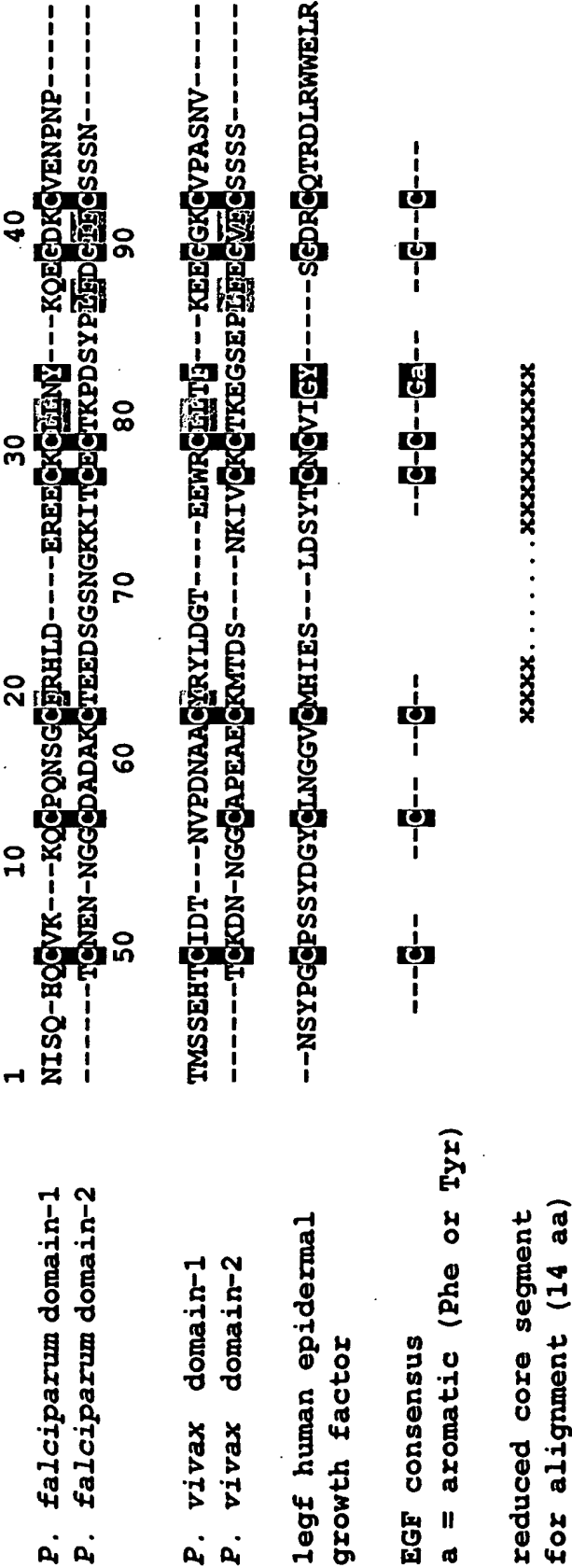


FIG. 1



2 / 17

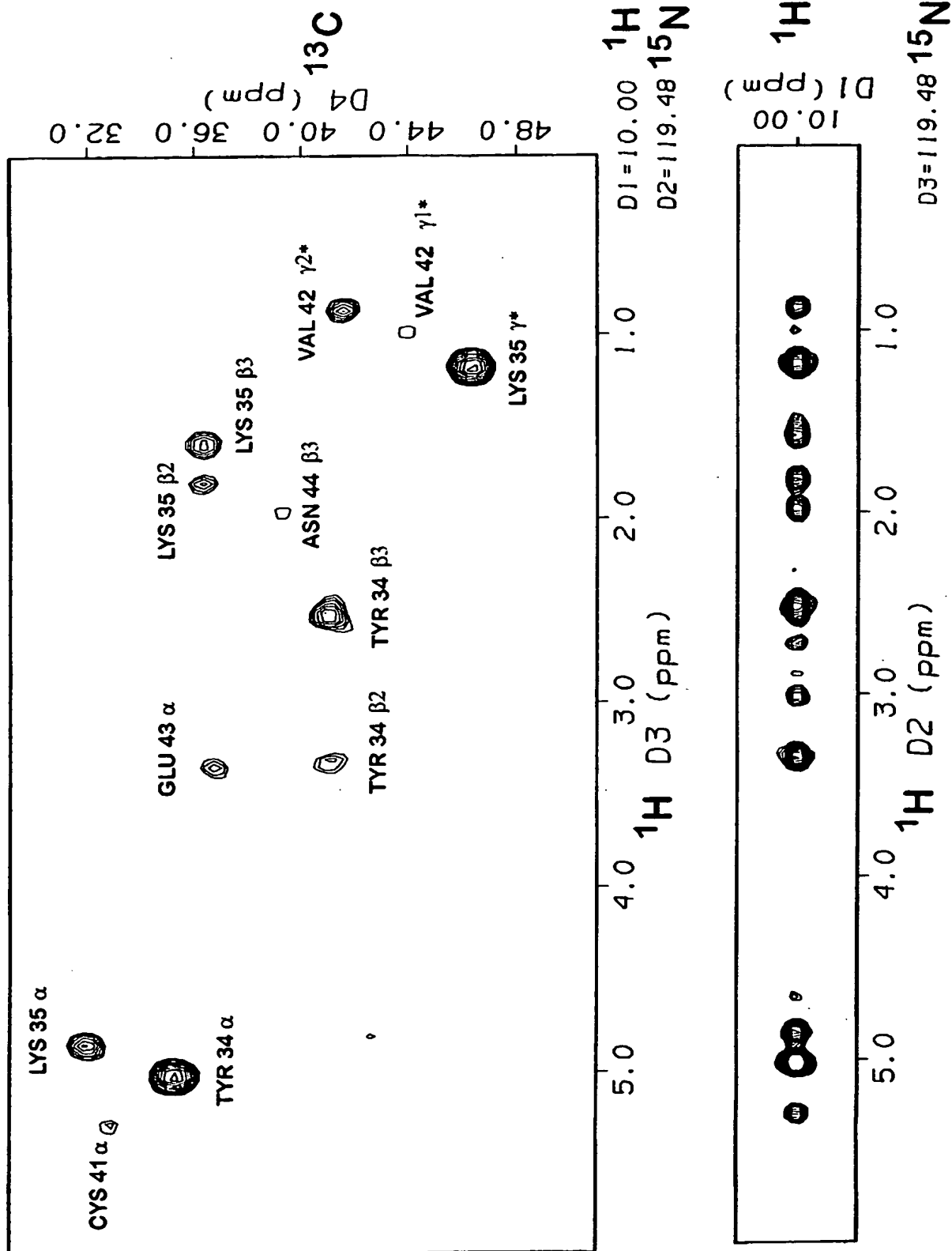


FIG. 2

3 / 17

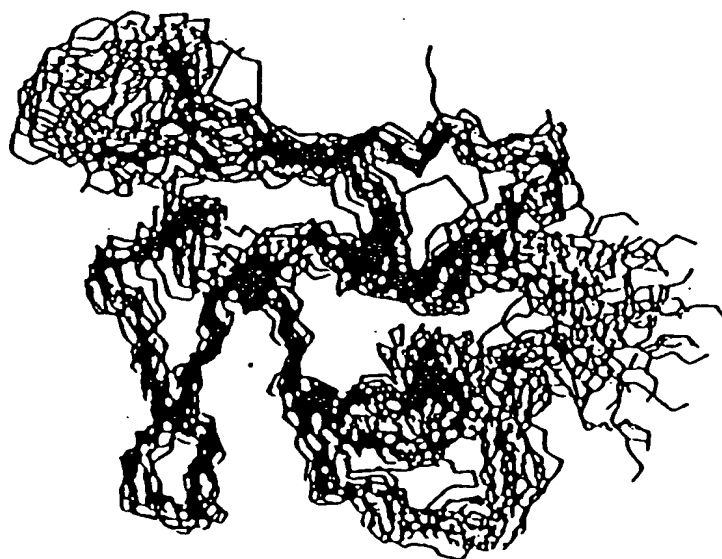
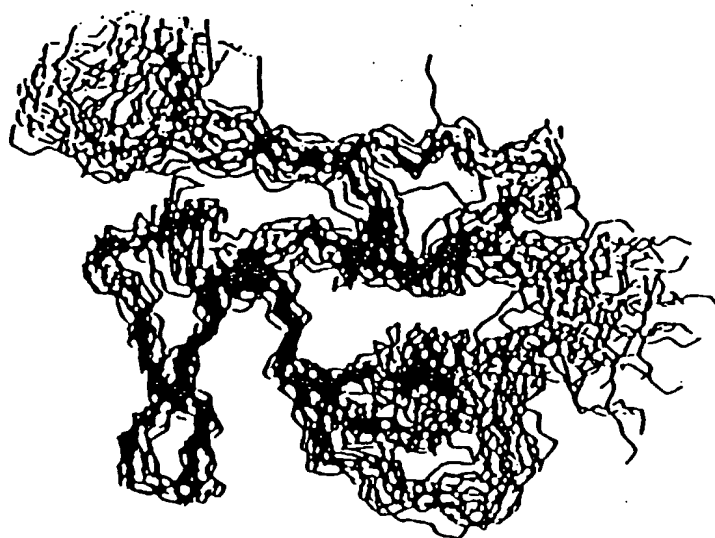


FIG. 3



FIG. 4

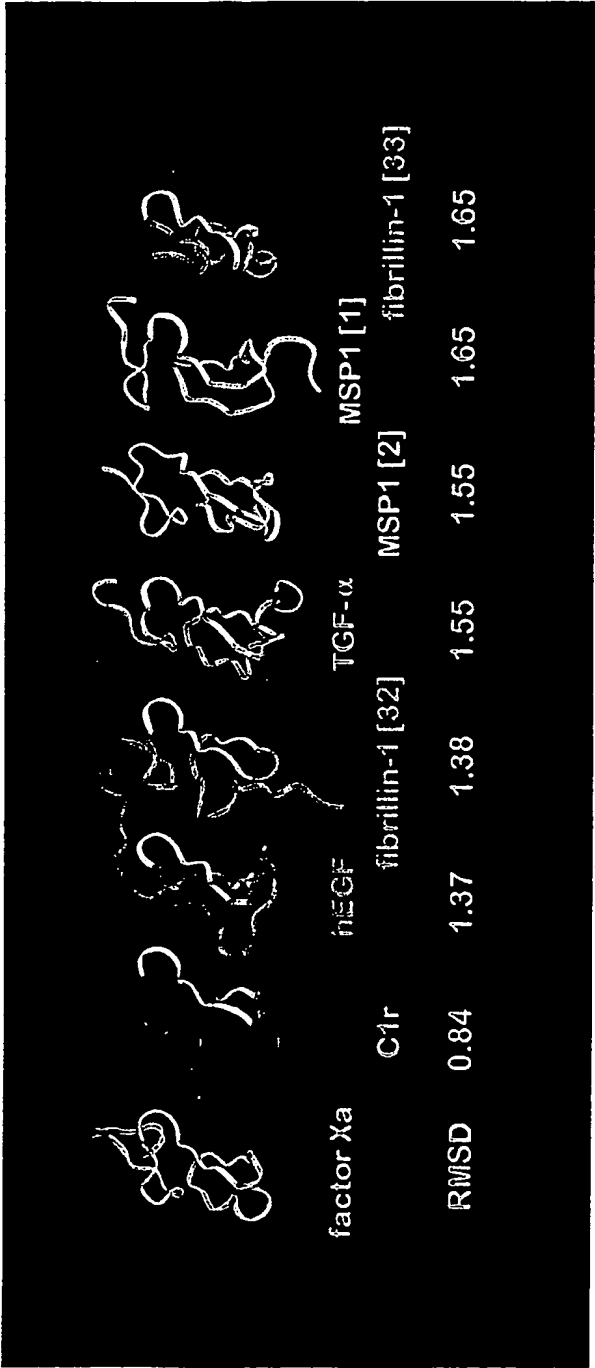


FIG. 5

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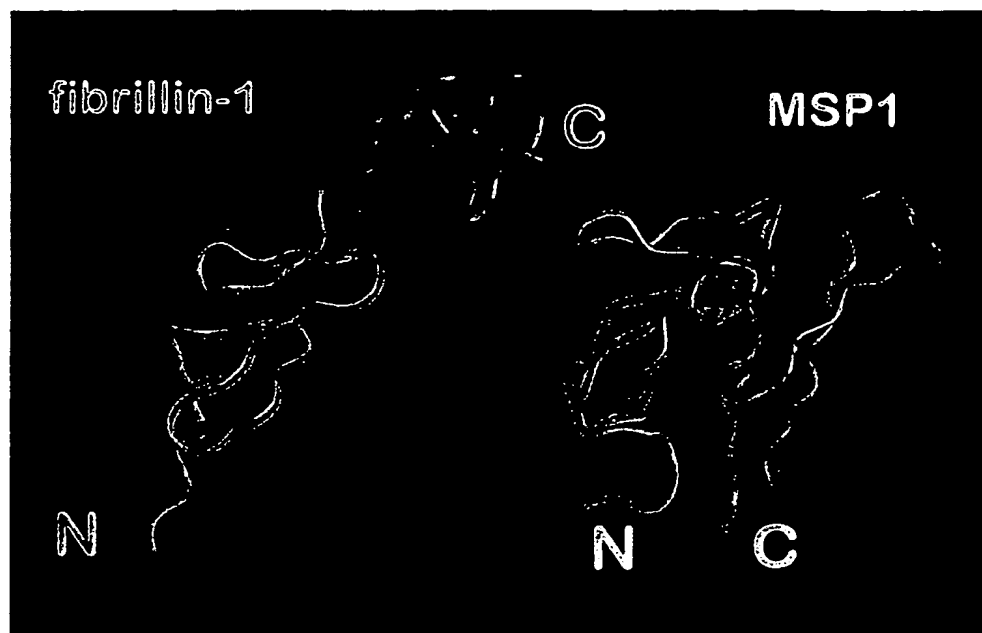


FIG. 6

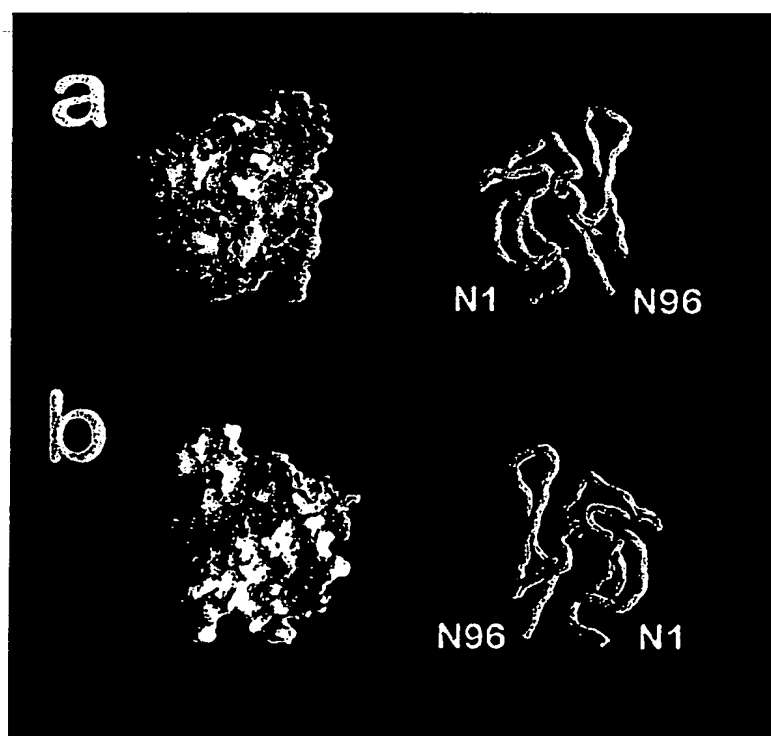


FIG. 7

SUBSTITUTE SHEET (RULE 26)

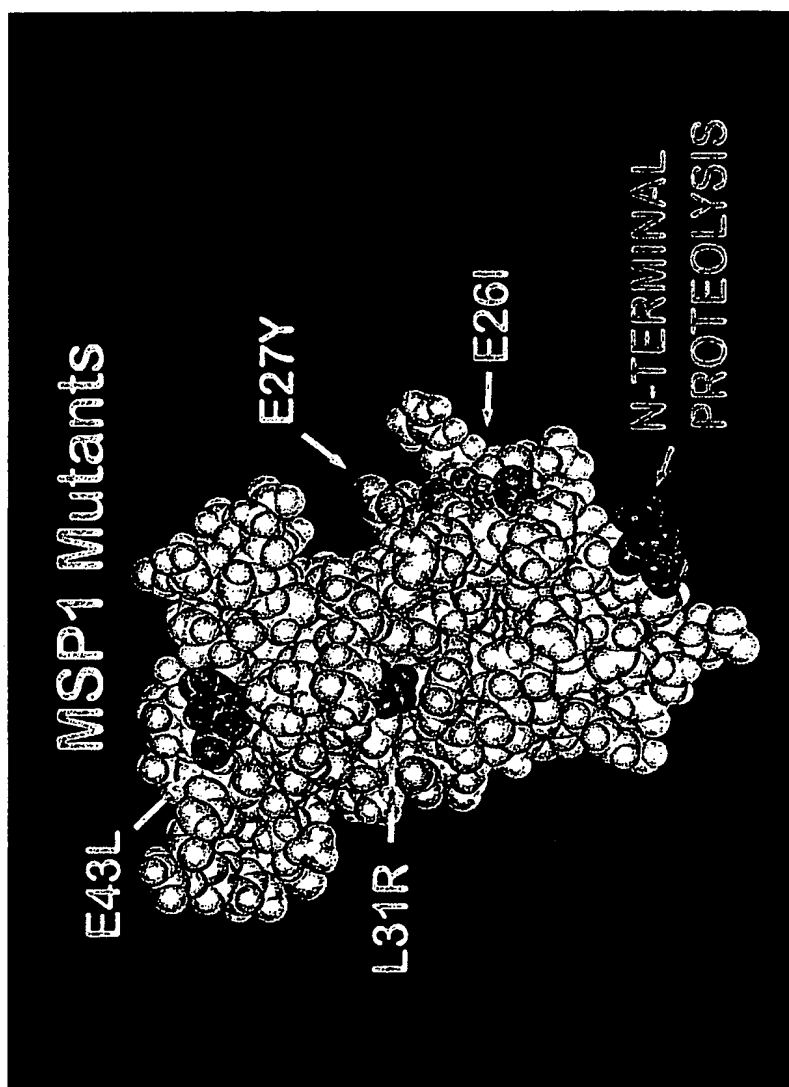


FIG. 8

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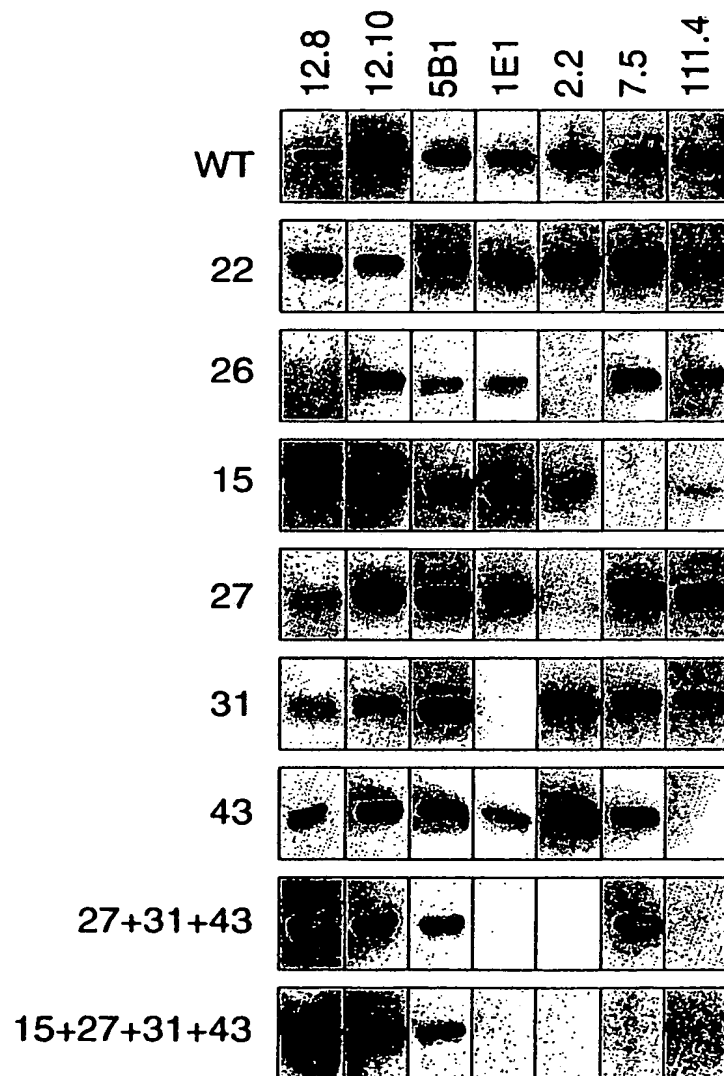
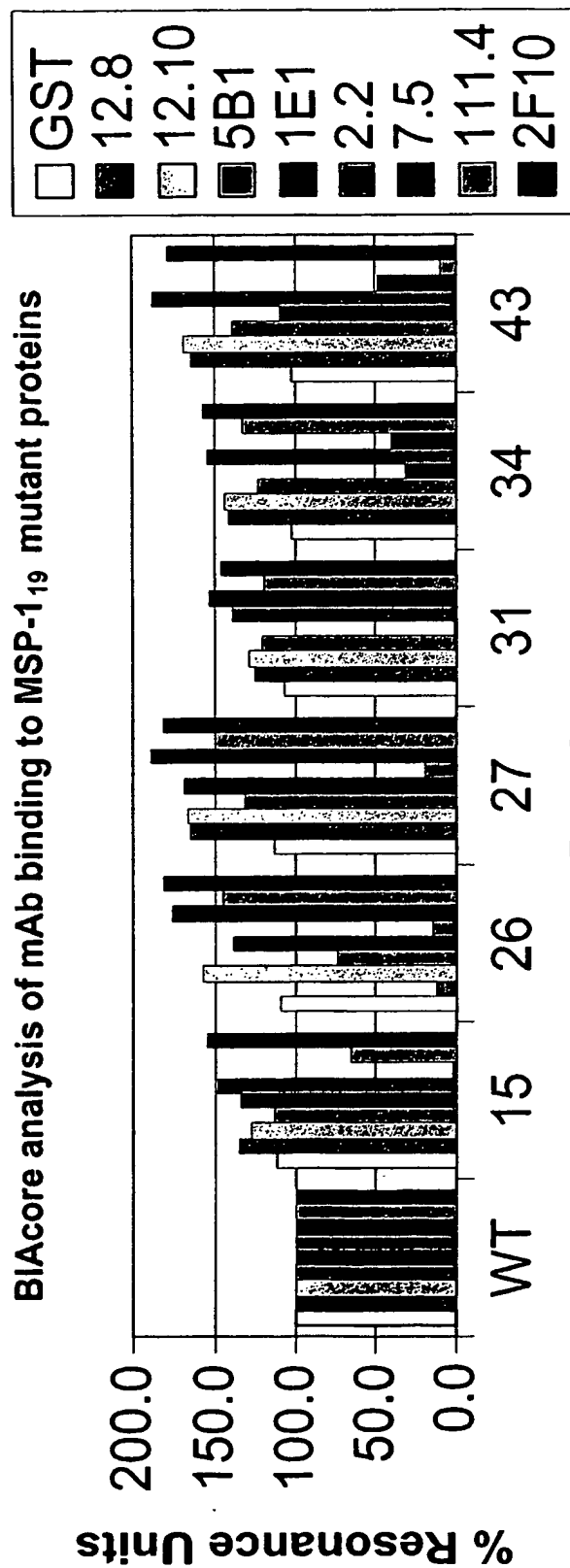


FIG. 9

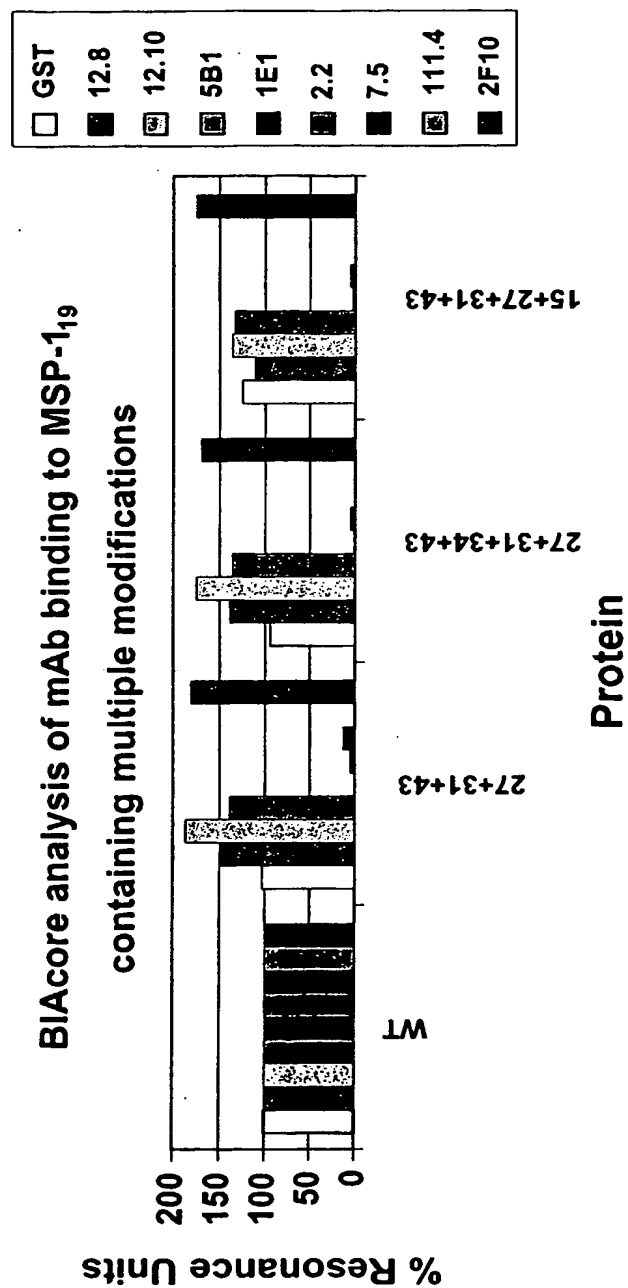
9 / 17



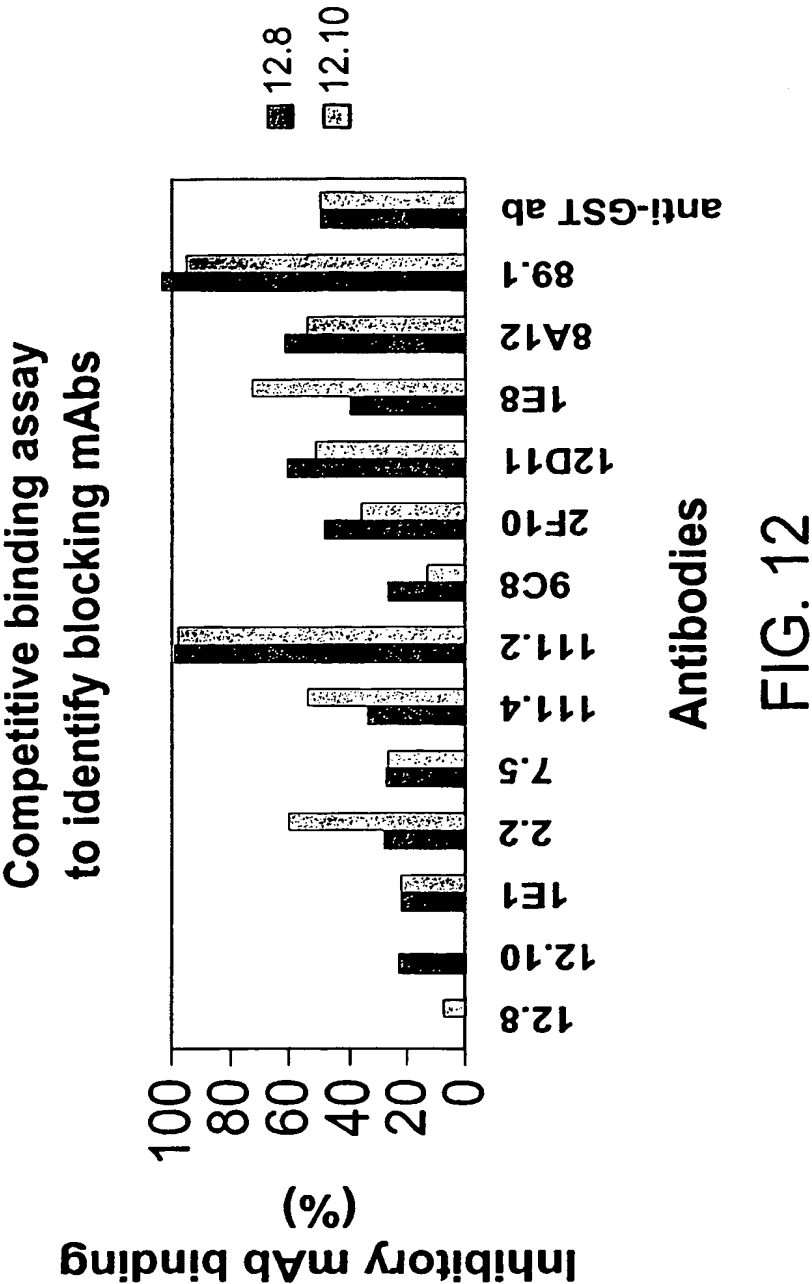
Protein

FIG. 10





**FIG. 11**



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### Effect of antibodies in the secondary processing assay

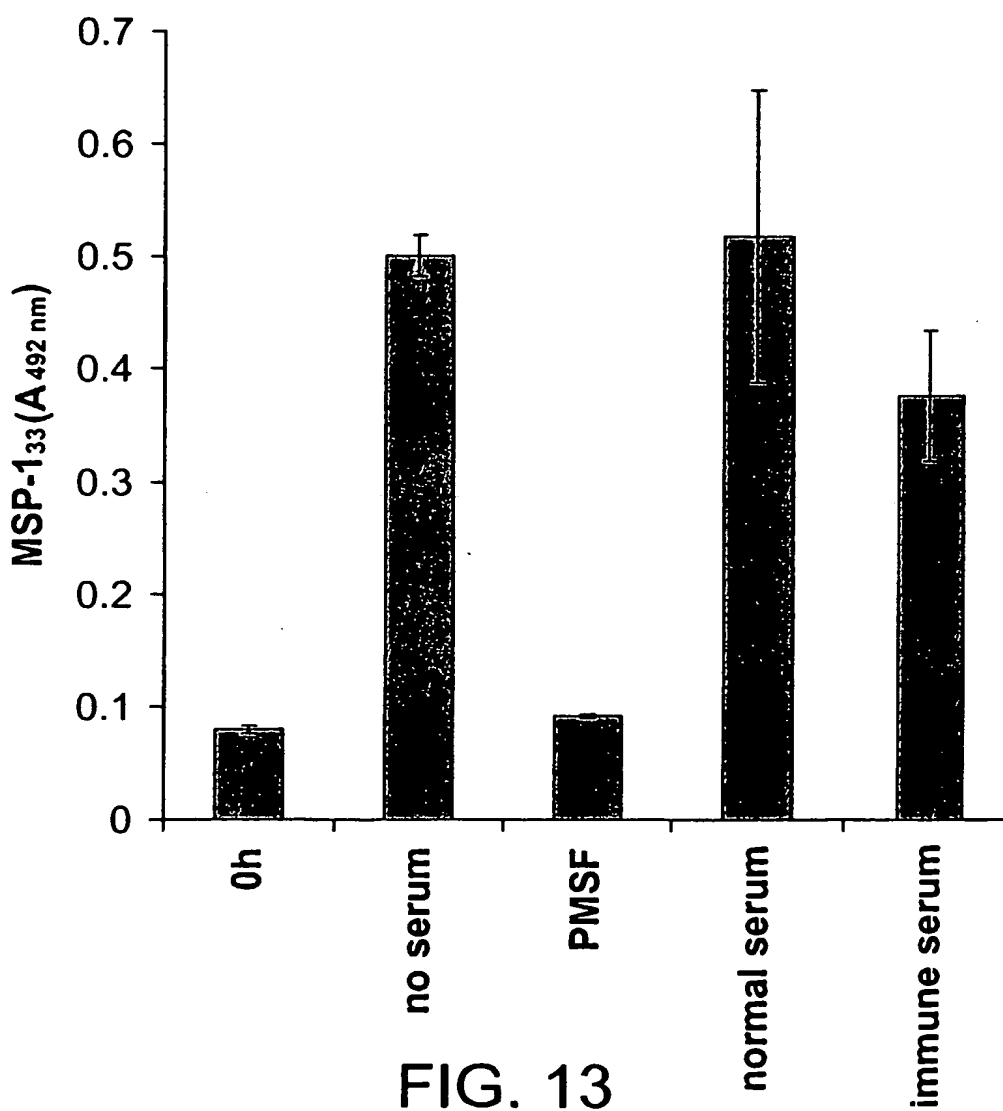


FIG. 13

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```

# Codon Usage Table for Komagataella (Pichia) pastoris
# for use with CODOP program
# Fields: [amino acid] [triplet] [frequency per thousand] [number]
# codon preferences weights:
#
#   30  preferred uniquely
#   20  preferred alternative
#   10  second best alternative
#   15  two equally preferred codons
#    1  unclassified
#    0  forbidden codons
#
# codon selection list
#
# Phe  TTC
# Leu  TTG > CTG
# Ile  ATC = ATT  and not  ATA
# Met  ATG
# Val  GTT > GTC  and not  GTA
# Ser  TCT = TCC
# Pro  CCA > CCT  and not  CCG
# Thr  ACT = ACC
# Ala  GCT > GCC  and not  GCG
# Tyr  TAC      and not  TAT
# *    UAA
# His  CAC > CAT
# Gln  CAA > CAG
# Asn  AAC
# Lys  AAG
# Asp  GAC > GAT
# Glu  GAA = GAG
# Cys  TGT > TGC
# Trp  TGG
# Arg  AGA      and not  CGC, CGA, CGG
# Gly  GGT > GGA  and not  GGG

```

Phe UUU 1.0( 1)	Ser UCU 15.0( 15)	Tyr UAU 0.0( 0)	Cys UGU 20.0( 20)
Phe UUC 30.0( 30)	Ser UCC 15.0( 15)	Tyr UAC 30.0( 30)	Cys UGC 10.0( 10)
Leu UUA 1.0( 1)	Ser UCA 1.0( 1)	Ter UAA 30.0( 30)	Ter UGA 0.0( 0)
Leu UUG 20.0( 20)	Ser UCG 1.0( 1)	Ter UAG 0.0( 0)	Trp UGG 30.0( 30)
Leu CUU 1.0( 1)	Pro CCU 10.0( 10)	His CAU 10.0( 10)	Arg CGU 1.0( 1)
Leu CUC 1.0( 1)	Pro CCC 1.0( 1)	His CAC 20.0( 20)	Arg CGC 0.0( 0)
Leu CUA 1.0( 1)	Pro CCA 20.0( 20)	Gln CAA 20.0( 20)	Arg CGA 0.0( 0)
Leu CUG 10.0( 10)	Pro CCG 0.0( 0)	Gln CAG 10.0( 10)	Arg CGG 0.0( 0)
Ile AUU 15.0( 15)	Thr ACU 15.0( 15)	Asn AAU 1.0( 1)	Ser AGU 1.0( 1)
Ile AUC 15.0( 15)	Thr ACC 15.0( 15)	Asn AAC 30.0( 30)	Ser AGC 1.0( 1)
Ile AUA 0.0( 0)	Thr ACA 1.0( 1)	Lys AAA 1.0( 1)	Arg AGA 30.0( 30)
Met AUG 30.0( 30)	Thr ACG 1.0( 1)	Lys AAG 30.0( 30)	Arg AGG 1.0( 1)
Val GUU 20.0( 20)	Ala GCU 20.0( 20)	Asp GAU 10.0( 10)	Gly GGU 20.0( 20)
Val GUC 10.0( 10)	Ala GCC 10.0( 10)	Asp GAC 20.0( 20)	Gly GGC 1.0( 1)
Val GUA 0.0( 0)	Ala GCA 1.0( 1)	Glu GAA 15.0( 15)	Gly GGA 10.0( 10)
Val GUG 1.0( 1)	Ala GCG 0.0( 0)	Glu GAG 15.0( 15)	Gly GGG 0.0( 0)

FIG. 14

SUBSTITUTE SHEET (RULE 26)

gctgttactccatctgttatcgataacattctgtctaagattggagaacgaatacagaggtcttbtgacttgaagcctctggc  
ggtgttactcagatccttgagaaacacgtgaaacacacgtacgtacattcaacgttaacgtcaaggacattttgaact  
ccagattcaacagagagaaattcaagacgttctggagctgacttgattcctcaagatttgactttcttcaac  
taagtttaaggaccatacaagttcctgaacaaggagaagagacaagattccttgcctcttacaatacatataagga  
ctccattgatactgatacaacttcgttaacgacgtcctgggttactacaagattctgtcgaagaagtaacaagcttgact  
tggattccatcaagaagtacatcaatgatagaagaagagaataaaaagtacctgccattcctgaaataacattgaaact  
ttgtacaagctgttaacgataaagatcgattcttcgtcattcttggagccaaagtttgcatacactacactacgaaaa  
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gttttcgagaacttggctaagacttctgtccaaactgttgatggtaacttgcaggttatgttgaaacttggccaaca  
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tgaaactaagacaggaaggtgataagttgttgagaaccaaacctacctgtaacgagacacaacggtggatgcgagct  
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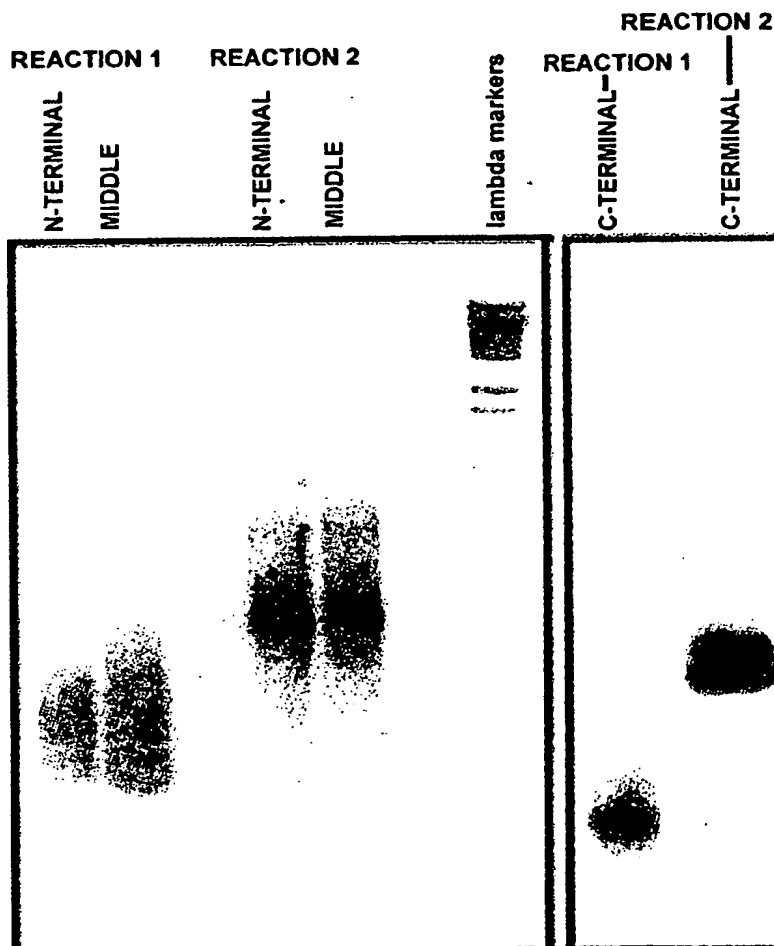
TACACCATCATCATCATCAATTGAAGGTAGACACAacattgccacaaccaatgcttaagaagcaatgtccacaaaactcgg  
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Accctacctgtaacgagacaacggtggatgcgcctgcgctaagtgcaccgaagaagactctggtctaacggaaaagaagatt  
acttgcgaatgtactaagccagactcttaccccttgttcgatggaatcttctgtctcttc  
taactaaGTGGTA

AKCTEEDSGSNGKKITCECTKPDSPYPLFDGIFCSSN.  
YHHHHHHHIEGRHNIAQHQCCKQCPQNSGCFRHLDEREECKLLNYKQEGDKCVENPNPTCNENNGGCDAD

gctgttactccatctgttatcgataacattctgtctaagattgagaacgaatacagaggtcttgtacttgaagcctctggc  
ccggtgtctacagatccctgagaagcaactgaaaacacgtcatgactttcaagttaacgtcaaggacattttgact  
ccagattcaacaagagaaaaacttcaagaacgttctggagctgacttgattccatacaaggatttgacttcttctaac  
tactgtttaaaggaccatacaagttcctgaacaggagaagagacaagtctctgtcctcttacaactacattaaagga  
tctcatctgatactgatacaacttcgttaacgagctcctgggttactacaagatcttgtcgagaagtaacaagtcgact  
cggattccatcaagaagtacatcaatgataagcaggagagatgaaaagtaacctgccattcctgataacattgaaact  
ttgtacaagactgttaacgataaagatcgactgttcgtcatctatgtgaagccaagcttgcatacacttacgaaaa  
gtctaacgtcgaggtcaagatcaaggaattgaactacctcaagaccattcaggataagctggctgatttcaagaagaca  
acaactcgttggatcgtgattgtccactgattacaacacaacaattctgttgactaagttcctgtctaacoggatg  
gttttcgagaacttggctaagactgtcctgtccaacctgttgatggtaacttgcagggttatgttg

AVTPSVIDNLSKIENEYEVLYKPLAGVYRSLKKQLENNVMTFNVNVKDILNSRFNKRENFKNVLES  
DLPYKDLTSSNVVVKDPYKFLNKEKRDKFLSSNYIKDSITDINFANDVLGYKILSEKYSKDLDSIKKY  
INDKQGENEKYLPFLNNIETLYKTVNDKIDLFVIHLEAKVLQYTEKSNVEVKIKELNYLKTIQDKLADFKKN  
NNNFVGIADLSTDYNHNNLLTKELSTGMVFENLAKTVLSNLLDGNLQGLML.

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**GENE ASSEMBLY PCR****FIG. 16**

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## EXPRESSION OF SYNTHETIC MSP1-19 GENE IN P. PASTORIS

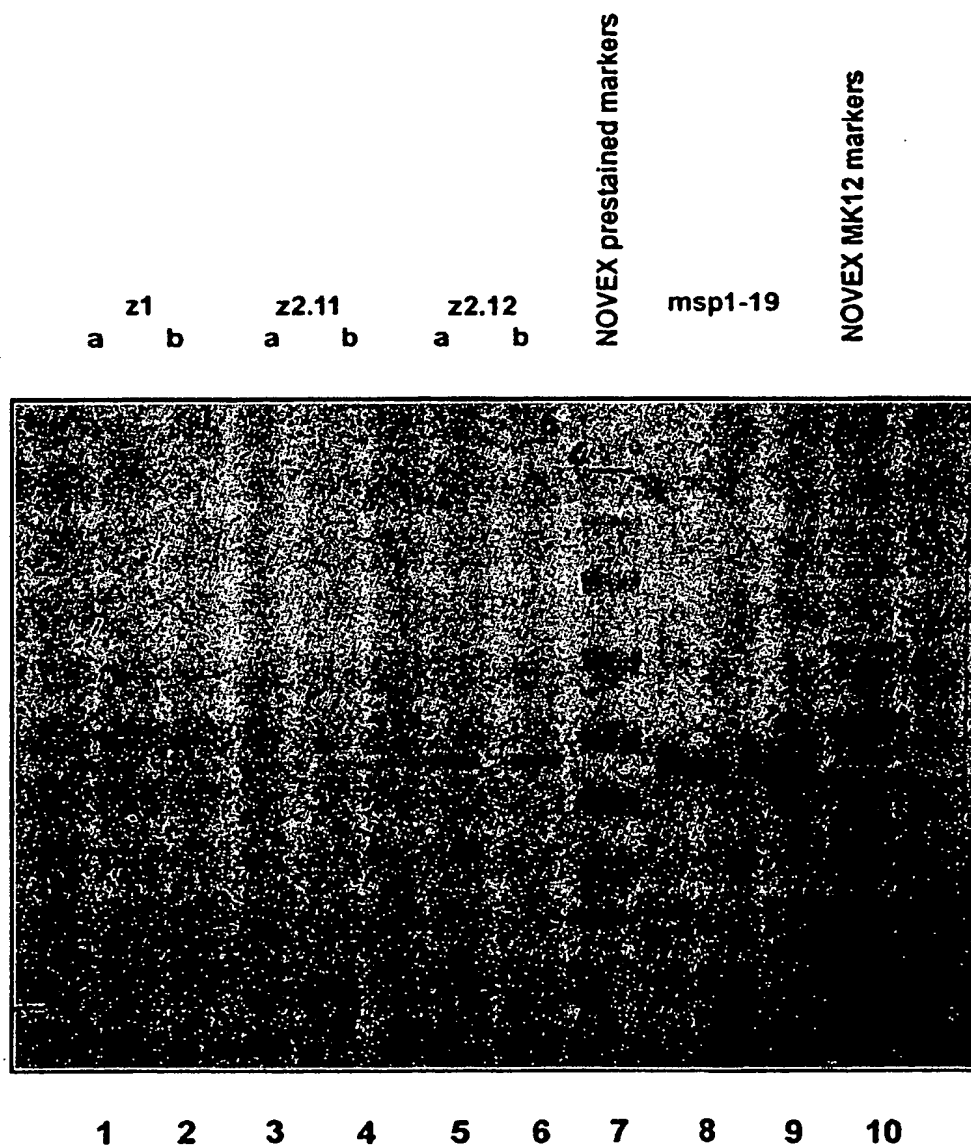


FIG. 17

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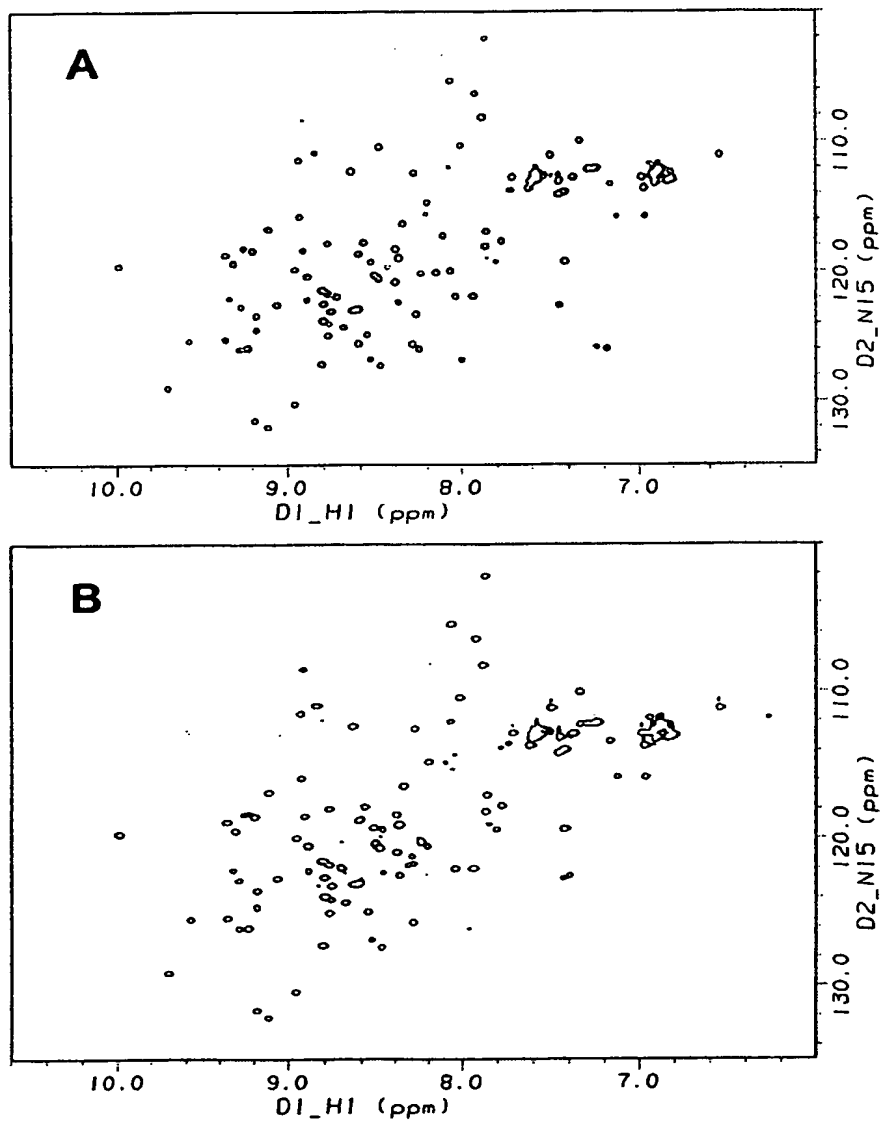


FIG. 18



## INTERNATIONAL SEARCH REPORT

national Application No

PCT/GB 00/01558

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/445 C12N15/30 C12N5/10 A61K39/00 C07K16/20  
G06F17/50 A61K49/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K A61K G06F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, STRAND, GENSEQ, CHEM ABS Data, MEDLINE, SCISEARCH  
EMBASE, EMBL

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 30158 A (NATO FARIDABANO ;ROTH CHARLES (FR); LONGACRE ANDRE SHIRLEY (FR); P) 21 August 1997 (1997-08-21)  page 14, line 13 -page 15, line 17 page 16, line 5-19 page 33, line 9-23 page 34, line 21-31	1-5, 13, 14, 16-27, 29, 33-36
Y	--- -/--	14-17, 30

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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- \* & \* document member of the same patent family

Date of the actual completion of the international search

4 December 2000

Date of mailing of the international search report

19/12/2000

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Authorized officer

ALCONADA RODRIG..., A

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KASLOW DAVID C ET AL: "Expression and antigenicity of Plasmodium falciparum major merozoite surface protein (MSP1-19) variants secreted from Saccharomyces cerevisiae." MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 63, no. 2, 1994, pages 283-289, XP000603953 ISSN: 0166-6851	1-5, 13-27, 29,33-36
Y	the whole document	14-17,30
X	--- DATABASE GENEMBL 'Online! 7 September 1993 (1993-09-07) JONGWUTIVES, S.: "P.falciparum DNA for the precursor to the major merozoite surface proteins, C-terminal" XP002154052 Accessions D13356 and D13345 -& JONGWUTIWES ET AL.: "Sequence conservation in the C-terminal part of the precursor to the major merozoite surface proteins (MSP1) of Plasmodium falciparum from field isolates" MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 59, 1 January 1993 (1993-01-01), pages 95-100, XP000603762	29-36
X	--- DATABASE GENEMBL 'Online! 24 March 1998 (1998-03-24) KANEKO, O.: "Plasmodium falciparum gene for merozoite surface protein 1, partial cds" XP002154053 Accession D86296 -& KANEKO ET AL.: "Plasmodium falciparum: Allelic variation in the merozoite surface protein 1 gene in wild isolates from southern Vietnam" EXPERIMENTAL PARASITOLOGY, vol. 86, no. 1, 1997, pages 45-57, XP000972338 the whole document	29-36
X	--- LONGACRE SHIRLEY ET AL: "Plasmodium vivax merozoite surface protein 1 C-terminal recombinant proteins in baculovirus." MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 64, no. 2, 1994, pages 191-205, XP000603954 ISSN: 0166-6851 page 192, right-hand column -page 193, left-hand column figures 5-8 --- -/--	29,33-36

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PAN WEIQING ET AL: "Vaccine candidate MSP-1 from Plasmodium falciparum: A redesigned 4917 bp polynucleotide enables synthesis and isolation of full-length protein from Escherichia coli and mammalian cells." NUCLEIC ACIDS RESEARCH, vol. 27, no. 4, 15 February 1999 (1999-02-15), pages 1094-1103, XP000953100 ISSN: 0305-1048 the whole document	29, 33-36
Y	page 1095, left-hand column, last paragraph	30
Y	--- VEDVICK T S: "GENE EXPRESSION IN YEAST: PICHIA PASTORIS" CURRENT OPINION IN BIOTECHNOLOGY, GB, LONDON, 1991, pages 742-745, XP000915577 ISSN: 0958-1669 the whole document	15-17, 30
Y	--- KOCKEN CLEMENS H M ET AL: "High-level expression of Plasmodium vivax apical membrane antigen 1 (AMA-1) in Pichia pastoris: Strong immunogenicity in Macaca mulatta immunized with P. vivax AMA-1 and adjuvant SBAS2." INFECTION AND IMMUNITY, vol. 67, no. 1, January 1999 (1999-01), pages 43-49, XP002153976 ISSN: 0019-9567 page 45, left-hand column -page 46, right-hand column, paragraph 1; figure 1	15-17, 30
A	--- PERERA K L R LAKSHMAN ET AL: "Baculovirus merozoite surface protein 1 C-terminal recombinant antigens are highly protective in a natural primate model for human Plasmodium vivax malaria." INFECTION AND IMMUNITY, vol. 66, no. 4, April 1998 (1998-04), pages 1500-1506, XP002153977 ISSN: 0019-9567 the whole document --- -/--	20, 23-26

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BLACKMAN MICHAEL J ET AL: "Antibodies inhibit the protease-mediated processing of a malaria merozoite surface protein." JOURNAL OF EXPERIMENTAL MEDICINE, vol. 180, no. 1, 1994, pages 389-393, XP000957584 ISSN: 0022-1007 cited in the application figures 2,3 page 391, left-hand column -page 392, left-hand column, paragraph 1 ---</p>	1-4
A	<p>PATINO JOSE A GUEVARA ET AL: "Antibodies that inhibit malaria merozoite surface protein-1 processing and erythrocyte invasion are blocked by naturally acquired human antibodies." JOURNAL OF EXPERIMENTAL MEDICINE, vol. 186, no. 10, 17 November 1997 (1997-11-17), pages 1689-1699, XP000957550 ISSN: 0022-1007 figures 1,2 ---</p>	1-4
P,X	<p>LALITHA P V ET AL: "Plasmodium falciparum: Variations in the C-terminal cysteine-rich region of the merozoite surface protein-1 in field samples among Indian isolates." EXPERIMENTAL PARASITOLOGY, vol. 92, no. 1, May 1999 (1999-05), pages 12-18, XP000957557 ISSN: 0014-4894 page 15, left-hand column, last paragraph -page 15, right-hand column; figure 2 ---</p>	1-5, 13-27, 29,30, 33-36
P,X	<p>MORGAN WILLIAM D ET AL: "Solution structure of an EGF module pair from the Plasmodium falciparum merozoite surface protein 1." JOURNAL OF MOLECULAR BIOLOGY, vol. 289, no. 1, 28 May 1999 (1999-05-28), pages 113-122, XP000957556 ISSN: 0022-2836 cited in the application see Materials and Methods ---</p>	28-30, 33-36
	<p>---</p> <p>-/--</p>	

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 00/01558

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>AUFIERO B M ET AL: "Epitope mapping of mAbs that inhibit or block secondary processing of MSP142." AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE, vol. 61, no. 3 SUPPL., 1999, pages 345-346, XP000957837 48th Annual Meeting of the American Society of Tropical Medicine and Hygiene; Washington, D.C., USA; November 28-December 2, 1999 ISSN: 0002-9637 abstract</p> <p>-----</p>	1-4

**FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210**

**Continuation of Box I.1**

Although claims 23-26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although claim 28 could be, at least partially, be considered as a mere presentation of information according to rule 39.1 PCT, the search has been carried out as far as possible in our systematic documentation.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 00/01558

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9730158 A	21-08-1997	FR 2744724 A	14-08-1997
		AU 1884197 A	02-09-1997
		CA 2241802 A	21-08-1997
		EP 0880588 A	02-12-1998
<hr/>			

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